# THE UNIQUE ACTIVATION STATUS OF INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

By

WALTER W ALLER, JR.

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

This work is dedicated to my mother, sister and father

ACKNOWLEDGMENTS

I wish to thank Drs. Richard Moyer, Richard Condit, Wayne McCormack, Joel Schiffenbauer and Gary Butcher for their support and guidance. I most sincerely wish to thank my mother, Ruth D. Aller, and sister, Helen E. Aller, without whose love, constant encouragement and support, both financial and emotional, none of this would have been possible. I also wish to thank my father, Walter W. Aller, for instilling in me the values and ideals that have made me who I am today.

TABLE OF CONTENTS

PAGE

ACKNOWLEDGMENTS ii

LIST OF FIGURES vii

ABSTRACT viii

CHAPTERS

1 INTRODUCTION 1

2 SIGNAL TRANSDUCTION IN T CELLS 2
TCR Structure 3
Protein Tyrosine Kinases (PTK) in TCR Signalling 8
Fyn 11
Lck 12
ZAP 70 14
MAP Kinases 16
CD45 18
Phospholipase C 19
Interactions of PTK in TCR Function 22
The Role of Calcium in T Cell Activation 24
Calcium Ion $Ca^{++}$ 24
Calcineurin 25

3 INTESTINAL INTRAEPITHELIAL LYMPHOCYTES 26
Phenotype 26
General 26
Repertoire Development 27
TAP and Beta-2 microglobulin 28
Hormone Effect on iIEL 28
CD Molecule Expression 29
Effects of Age 31
Other Models 31
Function 33
Expansion and Colonization in vivo 33
Proliferation and Cytokine Production 34
Signal Transduction 35
Cytotoxicity 36
Development 38
Adhesion Molecule Expression 41

4 MATERIALS AND METHODS 45
Animals 45
Reagents 45
iIEL Isolation 46
Proliferation Assays 46
Intracellular Calcium Measurement 47
Western Immunoblot Analysis 48
Granzyme B Assays 50

5 RESULTS 51
Chicken iIEL do not Proliferate in Response to Mitogens 51
Phenotype of Chicken iIEL 54
Granzyme B is Expressed at Higher Levels in Freshly Isolated iIEL than in PBLs 54
iIEL do not Mobilize Intracellular Calcium in Response to Calcium Ionophores 63
The Refractory Phenotype of iIEL is not due to Overstimulation by Exogenous Antigen in the Gut 71
Altered Tyrosine Phosphorylation of iIEL Intracellular Signalling Molecules 72
Specific Signal Transduction Molecules are Differentially Expressed or Phosphorylated in iIEL as Compared to Splenocytes. 81
MAP2 kinase 81
Fyn and ZAP-70 84

6 DISCUSSION 87
Chickens May Serve as Good Animal Model to Study iIEL 87
iIEL Constitutively Express Higher Granzyme B Levels than PBL 89
iIEL do not Mobilize Intracellular Calcium in Reponse to Calcium Ionophores 90
The Lack of in vitro Responsiveness is not due to Overstimulation by Exogenous Antigens. 91
iIEL Exhibit Different Protein Tyrosine Phosphorylation Patterns from Splenocytes 93
iIEL Express Different Signal Transduction Molecules from Splenocytes 95

7 CONCLUSIONS 98
IEL Function and Phenotype is Evolutionarily Conserved Between Chickens and Mammals 98
The Lack of Proliferation in Response to Stimulation by Specific and Nonspecific Mitogens May not be Due to Overstimulation by Exogenous Antigen 98
iIEL May Have Impaired Calcium Signaling Pathways 98
iIEL Have Altered Phosphorylation Patterns of Signal Transduction Molecules 98
iIEL Differentially Express Certain Signal Transduction Molecules 99

8 FUTURE DIRECTIONS 100

LIST OF REFERENCES ..... 105

BIOGRAPHICAL SKETCH ..... 117

LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| 1 | T cell receptor structure | 6 |
| 2 | Proximal signal transduction pathway in T cells | 10 |
| 3 | Distal signal transduction pathway in T cells. | 21 |
| 4 | Proliferation of chicken iIEL and splenic lymphocytes | 53 |
| 5 | Flow cytometric analysis of chicken duodenal iIEL | 58 |
| 6 | Flow cytometric analysis of chicken ileal iIEL | 60 |
| 7 | Flow cytometric analysis of chicken splenocytes | 62 |
| 8 | Granzyme B levels of iIEL and PBL | 65 |
| 9 | Calcium release by chicken lymphocytes | 68 |
| 10 | Calcium release by mouse lymphocytes | 70 |
| 11 | Proliferation of 20 day old chicken embryonic lymphocytes | 74 |
| 12 | Calcium release by 20 day old chicken embryonic lymphocytes | 76 |
| 13 | Western blot analysis of phosphotyrosine in mouse iIEL and splenocytes | 79 |
| 14 | Western blot analysis of MAP kinase of mouse iIEL and splenocytes | 83 |
| 15 | Western blot analysis of ZAP-70 and Fyn of mouse iIEL and splenocytes | 86 |

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

THE UNIQUE ACTIVATION STATUS OF
INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

By

Walter W. Aller, Jr.

May 1997

Chairman: Richard Moyar
Major Department: Molecular Genetics and Microbiology

Intestinal intraepithelial lymphocytes (iIEL) represent 25-40% of the total lymphocytes in the body of mice and humans. The study of this population of cells is often hindered by low yield of iIEL from mice. The possibility of using the chicken model for study of iIEL was investigated using phenotypic and functional studies. iIEL from chickens were found to have a similar phenotype to mouse iIEL as assessed by flow cytometry (52% αβ TCR+, 37% γδ TCR+, 87% CD3+, 8% CD4$^{+}$, 74% CD8$^{+}$) and similar functional characteristics to mouse iIEL. Chicken iIEL did not proliferate when treated with anti-CD3 antibody or phorbol myristyl acetate (PMA) plus ionomycin. Chicken iIEL did not release intracellular calcium in response to treatment with ionomycin or A23187. Chicken spleen cells and peripheral blood lymphocytes (PBL) did proliferate in response to anti-CD3 and PMA plus ionomycin. Chicken spleen and PBL released intracellular calcium in response to ionomycin and A23187. These data indicate that the chicken may serve an appropriate model for the study of iIEL.

The cause of the nonresponsiveness of iIEL has been thought to be the high antigenic load that these cells are exposed to in the intestinal environment. This was tested using iIEL from 20-day old chick embryos that had never been exposed to exogenous antigens. These iIEL exhibited the same lack of responsiveness in proliferation assays and in calcium release assays. These data suggest that the intestinal environment rather than antigenic load is responsible for the nonresponsive phenotype of iIEL.

Investigations into the molecular nature of this nonresponsiveness were made by examining the phosphorylation state and expression of molecules that are known to be involved in signal transduction from the T cell antigen receptor (TCR) to the nucleus and initiating transcription of genes involved in T cell activation. iIEL and splenocytes were stimulated by crosslinking components of the TCR with antibodies. Cell lysates were electrophoresed and probed with antibodies to phosphotyrosine and specific signal transduction molecules. Eight distinct differences were found in the phosphorylation pattern between iIEL and splenocytes. There also existed differences in expression of certain signal transduction molecules. ERK-1 was detectable only in iIEL whether stimulated or unstimulated. ERK 2 was expressed at similar levels in both iIEL and splenocytes. ZAP 70 was not detectable in iIEL. Fyn was expressed by both iIEL and splenocytes at similar levels. These data indicate that distinct differences exist in the signal transduction pathways between iIEL and other populations of lymphocytes (splenocytes and PBL) and that these differences may be responsible for the unique activation status of iIEL.

# CHAPTER 1
# INTRODUCTION

The immune system of the host is continuously exposed to numerous environmental antigens via the skin and mucosa. The total surface area of mucosa is at least 200 times larger than the skin and, in humans, is comparable to the surface area of a basketball court. Among these mucosal surface areas, approximately 80 % is represented by the small intestine. Intestinal intraepithelial lymphocytes (iIEL) reside between the epithelium and the lamina propria of the small intestine and comprise a unique population of T cells that differ from other lymphocytes in many ways. These cells are about as numerous as those lymphocytes in the spleen (50-100 x $10^6$ in mice), one of the primary lymphoid organs, and about half as numerous as the T lymphocytes in all peripheral lymphoid organs combined.

iIEL are functionally and phenotypically distinct from splenic and peripheral blood lymphocytes in many ways. Many of these differences and the underlying causes of these differences are poorly understood. The possible benefits of a greater understanding of this population of unique cells are many, including understanding oral tolerance, enhancing mucosal immunity and more fully understanding T-cell anergy. As the first set of immune system cells that encounters any ingested antigen, these cells warrant a greater understanding of their function and activation requirements.

# CHAPTER 2
# SIGNAL TRANSDUCTION IN T CELLS

Activation of T cells by antigen occurs because the TCR is able to bind antigen complexed to a MHC molecule and to transduce this extracellular binding event to intracellular signalling machinery. The generation of immune responses to most antigens occurs through the clonal activation of antigen-specific T lymphocytes. The ability of T cells to recognize and respond to antigenic stimuli is mediated through the T cell antigen receptor (TCR). Engagement of the TCR by antigens or other stimulatory ligands triggers a cascade of biochemical events which culminate in lymphokine secretion, T cell activation, proliferation and differentiation. The biochemical mechanisms that couple receptor binding to various intracellular events has been intensely investigated. Evidence indicates that specific protein phosphorylation by protein tyrosine kinases (PTK) represents the initial transduction event induced by TCR crosslinkage. Whereas several specific events and molecules that are involved in this process have been elucidated, many, if not most, parts of the process of signal transduction remain a mystery. Many new strategies, including utilizing chimeric receptors, transgenic animals, spontaneous mutations in both mice and man, and use of new animal models, have permitted much recent progress in understanding the structural and functional features of this multi-subunit receptor and the signal transduction cascade.

## TCR Structure

The TCR is responsible for recognizing antigens presented by cells in the context of major histocompatability complex (MHC) molecules Antigens are presented for immune surveillance by T cells as small (8-12 amino acids) peptides in the outermost groove of MHC class II (in the case of "professional" antigen presenting cells, (APCs) such as macrophages, dendritic cells and others) or MHC class I (on all other nucleated cells) MHC class II molecules usually present antigens derived from extracellular sources, such as bacteria, that have been phagocytized by the professional APCs. MHC class II molecules are also engaged by the coreceptor CD4 on "helper" T cells thus restricting recognition of MHC class II molecules to recognition by CD4 T cells MHC class I molecules usually present antigens from internally derived proteins such as self proteins or proteins from viruses or other intracellular pathogens that have invaded a cell MHC class I molecules are engaged by the coreceptor CD8 that is present on cytotoxic T cells at the same time that the TCR engages the antigen in the presentation groove This restricts MHC class I recognition to CD8 expressing T cells. So, MHC class II APCs interact with CD4 T cells to stimulate them to exert their effector function, which is to produce cytokines and initiate an humoral (B-cell or immunoglobulin) or cellular (T cell or cytotoxic) immune reponse T cells that express the CD8 molecule can interact with cells that express the MHC class I molecule (all cells in the body) and exert their effector function, which is to kill the recognized cell T cells are educated in thymus so that they do not generally recognize self antigens

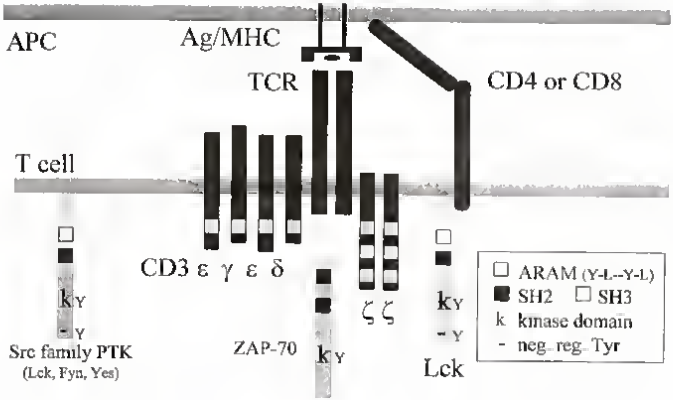
The TCR is a multi subunit complex consisting of α and β or γ and δ chains, the CD3 γ δ and ε chains, and a ζ–containing homodimer or heterodimer (Figure 1) Assembly of all subunits is required for efficient surface expression (Trowbridge, 1994, Weiss, 1986) The disulfide-linked α/β or γ/δ heterodimer is responsible for antigen/MHC binding (Dembic, 1986) and is noncovalently associated with the invariant CD3 and ζ chains The α, β, γ and δ chains are members of the immunoglobulin (Ig) superfamily and undergo gene rearrangement of V/J gene segments in the α and γ chain and V/D/J gene segments in the β and δ chain. This recombinatorial capacity of the αβ and γδ chains and the multiplicity of V, D, and J gene segments contribute to the marked diversity of the TCR so that it can accommodate the myriad of antigens that the immune system may encounter These chains, however, have only short cytoplasmic domains of five amino acids and are unlikely to be able to transduce extracellular binding events directly to the intracellular signalling machinery

The invariant CD3 chains, composed of γ, δ and two ε subunits, have extracellular Ig-like domains and in contrast to the TCR chains contain larger cytoplasmic domains, ranging from 40 to 80 amino acids The TCR and CD3 subunits are noncovalently associated with a zeta-zeta dimer The ζ chain is a transmembrane protein with a 9 amino acid extracellular domain, a single transmembrane domain, and a cytoplasmic domain of 113 amino acids (Weissman 1988 Bamyash,1989) Circular-dichroism analysis reveals a random coil conformation

The TCR initiates signals by interacting with cytoplasmic protein tyrosine kinases (PTKs) through a 17-residue sequence motif called the antigen recognition activation

Figure 1

T cell receptor structure and associated protein tyrosine kinases. Filled boxes represent Src homology 2 domains. Open boxes represent Src homology 3 domains. Shaded boxes represent antigen recognition activation motifs. k represents kinase domains. (–) represent negative regulatory tyrosine residues.


APC
Ag/MHC
TCR
CD4 or CD8
T cell
CD3 ε γ ε δ
ζ ζ
kY
Y
Src family PTK
(Lck, Fyn, Yes)
ZAP-70
kY
kY
Y
Lck
ARAM (Y-L--Y-L)
SH2
SH3
k kinase domain
- neg_reg_Tyr

motif (ARAM) that is contained in the TCR ζ and CD3 chains (Iwashima, 1993)

Analysis of the protein sequences present in the cytoplasmic domains of the ζ chain and the CD3 ε, γ and δ chain has revealed the presence of a common motif consisting of two YXXL paired sequences separated by six to eight amino acids (Kim, 1993) This ARAM sequence ( also called immunoreceptor tyrosine-based activation motifs or ITAMs) appears in triplicate in the ζ chain and is present as a single copy in the CD3 ε, γ and δ subunits Because each TCR can contain a TCRζ dimer and two CD3 dimers (εγ and εδ) each TCR can contain a total of 10 ARAMs This large number of ARAMs within a single complex may serve to amplify the signal received by each TCR

The importance of the two YXXL sequences is underscored by mutational analysis in which alterations of either tyrosine residues in an IL-2Rα ζ chimera resulted in a marked reduction of receptor function (Letourneur, 1992) In addition, deletion of the C-terminal leucine residue within the motif also resulted in loss of receptor function (Irving, 1993)

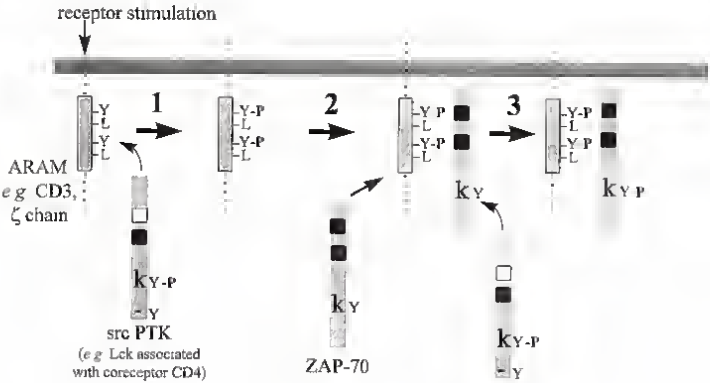
Recent evidence has also suggested a potential function for the cytoplasmic domain of the CD3 subunits in TCR signaling (Wegener, 1992) This finding suggested that a TCR component other than the ζ chain could provide signal transduction function. However antigen-induced IL-2 secretion by this receptor devoid of the cytoplasmic domain of ζ was reduced 10-fold relative to the wild type receptor (Hermans 1993) Further dissection of the CD3 subunits utilizing a chimeric receptor strategy revealed that the cytoplasmic domain of CD3 ε can also mediate TCR function (Letourneur, 1992)

Protein Tyrosine Kinases (PTK) in TCR Signalling

Unlike some transmembrane growth factor receptors that have intrinsic kinase activity, none of the polypeptides of the multi-subunit TCR contain intrinsic enzymatic activity. However, engagement of the TCR by antigen or anti-TCR antibodies activates a PTK pathway resulting in tyrosine phosphorylation of a variety of cellular proteins (Samelson, 1986, Hsi 1989). Upon TCR activation, the tyrosine residues within the ARAMs become phosphorylated, permitting the binding of Src homology 2 (SH2) domain-containing proteins (Figure 2). Additional signalling molecules are subsequently recruited to these newly TCR-associated proteins via SH2 or other modular interaction domains. At least three of the cytoplasmic PTKs, Fyn, Lck and ZAP-70 have been implicated in proximal TCR signaling. These cytoplasmic PTKs fall into two distinct categories of PTKs which differ structurally. Fyn and Lck are members of the Src family and ZAP-70 is a member of the Syk family. The Src-family PTKs share a number of common structural features that include (i) an N-terminal myristylated glycine at residue 2 that permits membrane localization, (ii) a unique ~80 amino acid N-terminal region that may dictate specific associations of the kinase, (iii) a ~60 amino acid Src-homology 3 (SH3) domain involved in interacting with signaling molecules with proline-rich regions (iv) an ~ 100 amino acid SH2 domain that can specifically mediate the association with tyrosine phosphoproteins, (v) a C-terminal catalytic domain, and (vi) a negative regulatory tyrosine residue C-terminal to the kinase domain. In contrast to the Src-family PTKs, the Syk-family PTKs contain two tandemly arranged SH2-domains and a C-terminal kinase domain (Chan, 1992). However, the Syk-family PTKs are not myristylated, do not

Figure 2

Proximal signal transduction pathway in T cells  Note that the ARAM of the TCR is first phosphorylatad by Lck, then the unphosphorylated form of ZAP-70 docks to the ARAM via SH2 domains  ZAP 70 is then also phophorylated by Lck to an activated state


receptor stimulation
ARAM
e g CD3,
ζ chain
Y
L
Y
L
1
Y-P
L
Y-P
L
2
Y P
L
Y-P
L
3
Y-P
L
Y P
L
kY-P
Y
src PTK
(e g Lck associated
with coreceptor CD4)
kY
ZAP-70
kY
kY-P
Y
kY P

contain SH3 domains, nor do they contain known sites of negative regulation at their C-terminus. When phosphorylated using recombinant Lck, ζ associates with recombinant ZAP-70 tandem-SH2 domains. All three T cell activation motifs in ζ bind ZAP-70 tandem-SH2 domains in vitro, forming a 1:3 complex, providing a mechanism for signal amplification. (Weissenhorn, 1996)

Fyn

Fyn is a 59 kDa src-family PTK expressed predominantly in tissues of neuronal and hematopoietic origin (Semba, 1986, Kawakami, 1986). The co-immunoprecipitation of Fyn with the TCR ζ chain was the first demonstration of an association of a PTK with the TCR complex (Samelson, 1990). The interaction of Fyn and the cytoplasmic domain of ζ appears to be mediated by the first 10 amino acids within the unique region of Fyn and at least two regions within the cytoplasmic domain of ζ that each encompass at least one ARAM sequence (Gauen, 1992). Chimeric receptors consisting of only one ARAM do not interact with Fyn. The association of Fyn with an ARAM does not appear to be absolutely required for TCR function. Rather, the association may play a role in augmenting TCR function.

Expression of a kinase-inactive Fyn molecule in transgenic mice abrogated TCR-mediated proliferation and mobilization of cytoplasmic calcium. Mice that lack Fyn as a result of homologous recombination demonstrated marked signalling abnormalities in the single positive thymocyte populations (Appleby, 1992, Stein, 1992). These single positive thymocytes showed diminished anti-TCR antibody-mediated $Ca^{++}$ mobilization as well as mitogenic lectin or anti-CD3 antibody-induced T cell proliferation. In contrast, peripheral T cells in these mice exhibited less pronounced effects. Splenic T cells deficient in Fyn

demonstrated decreased $Ca^{++}$ mobilization and IL-2 production, but exhibited normal proliferative responses to anti-CD3 antibody-mediated cross-linking, allogeneic cells, and to Staphylococcal enterotoxin A. Fyn also does not appear to be required for normal thymocyte development because the Fyn-deficient mice have grossly normal thymocyte subsets and normal TCR V β repertoires

Lck

Lck is a 56 kDa lymphoid-specific src-family PTK that noncovalently associates with the cytoplasmic domains of CD4 and CD8 (Rudd, 1988, Veillette, 1988, Turner 1990, Shaw, 1989, Shaw, 1989). This association of CD4 and CD8 with Lck has also suggested a potential role in signal transduction for these TCR coreceptors (Veillette, 1989). The association of CD4 with Lck has been demonstrated using a CD4-dependent antigen-specific murine T cell that lacks endogenous CD4 or CD8 (Glaichenhaus, 1981). Restoration of TCR function occurred only after the introduction of CD4 molecules that are capable of associating with Lck. The cytoplasmic domain of the CD8α chain has also been implicated using a murine T cell in which the full-length CD8α chain is required for optimal TCR function. Expression of a truncated form of CD8α abrogated function, suggesting that the lack of association of Lck to the cytoplasmic domain of CD8α may be contributing to this defect (Zamoyska, 1989, Letourneur, 1990).

Lck is also important in lymphocyte development. Mice deficient in Lck have a pronounced arrest in thymocyte development with a 10- to 30- fold decrease in total thymocyte number (Molina, 1992).

The role of Lck in TCR-mediated signaling was studied in a mutant of the Jurkat leukemic T cell line. This cell line, lacking a functional Lck PTK, fails to mobilize $Ca^{++}$, to

induce tyrosine phosphoproteins, or to express activation antigens following TCR stimulation (Strauss, 1992) Reconstitution with wild type murine Lck in this mutant restored all TCR-mediated functions (Karnitz, 1992)

Analysis of the domains within Lck that participate in TCR function demonstrated that membrane localization and the SH2 domain of Lck are both required (Caron, 1992) Mutation of the N-terminal site of myristylation preventing membrane localization of Lck Deletion of the SH2 domain of Lck abolished the TCR-induced tyrosine phosphorylation and antigen-induced IL-2 production (Abraham, 1990) Myristylation of Lck functions to localize this PTK to the plasma membrane, in close proximity to surface TCRs The SH2 domain of Lck may be required to interact with downstream effector molecules in propagating TCR function The SH2 domain of Lck confers additional information that may be required for CD4 coreceptor function (Xu, 1993)

These functional effects of Lck on TCR signaling suggest that components of the TCR complex may interact with Lck (Anderson, 1988, Mittler, 1989) A physical association between CD4 and TCR complexes has been demonstrated using a sensitive in vitro kinase assay, revealing the coimmunoprecipitation of CD3 ε and the CD4-Lck complex (Burgess 1991)

Extensive cross-linking of the TCR in the J CaM1 6 Lck-deficient mutant can elicit a TCR response, albeit diminished, suggesting that other PTKs such as Fyn may mediate functions that are typically served by Lck (Straus, 1992)

Activation of CD28 on T lymphocytes also initiates a cascade of intracellular events which, in concert with activation of the TCR, culminates in production of cytokines and a functional immune response. One of the earliest biochemical changes observed

following stimulation of CD28 is tyrosine phosphorylation. It was demonstrated that Lck was activated following cross-linking of CD28 (Gibson, 1996)

Ligation of CD44 by monoclonal antibodies transduces signals to T cells which lead to tyrosine phosphorylation of ZAP-70 and other intracelluler proteins. In vitro kinase assays demonstrate that cross-linking of CD44 induces an increase in the intrinsic activity of Lck. Immunoprecipitations show that CD44 is physically associated with Lck (Taher, 1996)

Recent results suggest that the activity of the Lck kinase domain in vivo is controlled by dephosphorylation at the negative regulatory site and phosphorylation at the positive regulatory (autophosphorylation) site ( Xu, 1995)

Using Lck SH2 and SH3 domains linked to glutathione S transferase, it was demonstrated that the SH2,3 region interacts specifically and directly with numerous tyrosine-phosphorylated molecules following TCR cross-linking, including constitutively with mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) and inducibly with the ζ chain of the TCR. The interaction with MAPK/ERK kinase was via the SH3 domain. The interaction with the tyrosine-phosphorylated ζ chain, while phosphotyrosine-dependent, required both the SH3 and SH2 domains (August, 1996)

ZAP 70

A third PTK implicated in proximal TCR signalling is ZAP-70. ZAP-70 (ζ-chain associated protein) was initially identified as a 70 kDa tyrosine phosphoprotein that associated with the TCR ζ chain following TCR stimulation (Chan, 1991). The ZAP-70 protein tyrosine kinase is essential for TCR-mediated signaling. The absence of ZAP-70 results in impaired differentiation of T cells and a lack of responsiveness to antigens

stimulation Mutations in ZAP-70 have been shown to be responsible for a rare, autosomal recessive form of severe combined immunodeficiency (SCID) in humans This distinctive SCID syndrome is characterized by the selective absence of peripheral CD8⁺ T cells and by abundant circulating CD4 T cells that fail to respond to TCR mediated stimuli in vitro (Elder 1995) Two ZAP-70 mutants devoid of kinase activity, generated either by a point mutation in the kinase domain to create an inactive kinase, or by truncation of the entire kinase domain functioned as dominant negative mutants to specifically suppress TCR mediated activation of the nuclear factor of activated T cells (NF-AT), a transcription factor essential for inducible interleukin 2 gene expression (Qian, 1993)

Several proteins have been tested as potential in vitro substrates of ZAP-70 Alpha tubulin and the cytoplasmic fragment of human erythrocyte band 3 (cb3), which have a region of sequence identity at the phosphorylation site, proved to be good substrates. In contrast to Lck, ZAP-70 did not phosphorylate the cytoplasmic portion of the TCR ζ chain or short peptides corresponding to the CD3ε or the TCR ζ ARAM indicating that ZAP-70 exhibits a high degree of substrate specificity (Isakov, 1994)

Unlike Fyn and Lck, the association of ZAP-70 with the TCR requires receptor stimulation In addition to ζ, the CD3 ε subunit can also associate with ZAP-70 following TCR stimulation (Wange, 1992, Straus1993) The association of ZAP-70 with the TCR demonstrates marked specificity (Chan, 1991) In a cell expressing two forms of the ζ chain, the multisubunit TCR and the CD8/ζ chimera, stimulation of the former results in association of ZAP-70 solely with the multi-subunit receptor, while stimulation of the latter results in the association of ZAP-70 solely with the chimeric receptor

Recent evidence suggests that the function of ZAP 70 and Syk PTKs may be differentially regulated (Kolanus, 1993) Cross-linking of chimeras consisting of a CD16 extracellular domain fused to ZAP-70 or Syk induced $Ca^{++}$ mobilization. Whereas cross-linking of the Syk chimera resulted in cytolysis, the ZAP-70 chimera required cross-linking with a Fyn or Lck chimera to effect cytolysis

The threshold at which antigen triggers lymphocyte activation is set by the enzymes that regulate tyrosine phosphorylation Upon T cell activation, the protein tyrosine phosphatase SHP-1 was found to bind to the protein tyrosine kinase ZAP-70 This interaction resulted in an increase in SHP 1 phosphatase activity and a decrease in ZAP-70 kinase activity Expression of a dominant negative mutant of SHP-1 in T cells increased the sensitivity of the antigen receptor Thus, SHP-1 functions as a negative regulator of the T cell antigen receptor and in setting the threshold of activation (Plas, 1996)

MAP Kinases

The MAP kinases (mitogen-activated proteins, microtubule associated proteins) are serine/threonine kinases that are stimulated upon TCR crosslinking. The members of this family, ERK1 (43kD), ERK2 (41kD) and ERK4 (45kD) (Extracellular Regulated Kinase) have been shown to cross the nuclear membrane and regulate transcription by interaction with the fos and jun transcriptional activators These proteins are present in many different cell types and are capable of mediating different activation patterns depending on the cells in which they are expressed

It was demonstrated that the SH2 and SH3 regions of Lck interact specifically and directly with MAPK/ERK The interaction with MAPK/ERK was via the SH3 domain.

The interaction with MAPK would place Lck in a position to be involved in the complex resulting in the activation of MAPK (August, 1995)

Using the T cell leukemic cell line Jurkat as a model for T cell activation, it was demonstrated that cross-linking CD28 using monoclonal antibodies causes tyrosine phosphorylation and activation of MAP kinase/ERK Activation of MAPK/ERK occurred 3-fold less efficiently in a Jurkat line lacking functional Lck and was almost undetectable in a line lacking CD45 (August, 1995)

ERK-1 and ERK 2 and the guanosine triphosphate-binding protein p21ras were found to remain unactivated upon stimulation of anergic murine T helper cell 1 clones (Fields 1996) In peripheral blood T cells, ERK2 was more prevalent than ERK1 The concentrations of ERK1 and ERK2 were not altered by stimulating the cells for 16 h with immobilized anti-CD3 mAb or anti-CD3 mAb and phorbol myristate acetate. Anti-CD3 antibodies and phorbol myristate acetate stimulated an increase in ERK1 and ERK2 kinase activity The data demonstrate that ERK1 and ERK2 are part of an anti-CD3 mAb-stimulated signal transduction cascade that is downstream of protein kinase C and suggest that these kinases play an important role in T cell activation (Whitehurst, 1992)

In mice expressing dominant-negative Ras and Mek 1 transgenes simultaneously, thymocyte maturation beyond the CD4 8 stage is blocked almost completely, whereas negative selection is quantitatively intact This suggests that activation of the MAPK cascade is necessary for positive selection, but irrelevant for negative selection (Alberola, 1996)

Other data suggest that the production of 3-phosphorylated inositol phospholipids is involved in the activation of ERK-2, but does not regulate the enzymes that are thought to be upstream of ERK-2

CD45

CD45 is one of the most abundant leukocyte cell surface glycoproteins and is expressed exclusively upon cells of the hematopoietic system CD45 was initially identified as a protein tyrosine phosphatase (PTP) This discovery linked CD45 to the process of reversible protein tyrosine phosphorylation CD45 is now established as a critical component of the signal transduction machinery of lymphocytes Evidence indicates that CD45 plays a pivotal role in antigen stimulated proliferation of T lymphocytes and in thymic development Structure-function analysis of CD45 has identified structural features of the PTP catalytic domains required for enzymatic activity The external domain of CD45 does not appear to be absolutely required for signal transduction by the T cell receptor (Trowbridge, 1994)

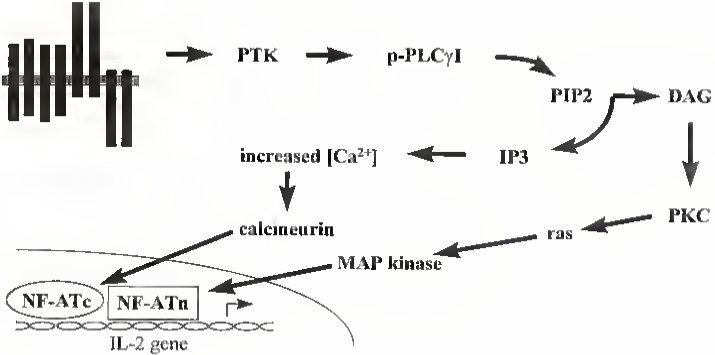
CD45 plays an essential role in T cell activation by activating Lck and/or Fyn Numerous experiments have indicated that CD45 may have both stimulatory and inhibitory roles in T cell activation. The complex regulation of the alternative splicing of the extracellular domain in various leukocyte lineages also suggests additional roles for the CD45 PTPase. The CD3 ζ chain is also a substrate of the CD45 PTPase Phosphorylated CD3 ζ chain was preferentially dephosphorylated by the wild-type CD45 PTPase under conditions that did not significantly dephosphorylate other cellular proteins Results suggest that CD45 is involved in the termination of the T cell response via dephosphorylation of CD3 ζ chain (Furukawa, 1994)

Phospholipase C

One of the cellular proteins that undergoes tyrosine phosphorylation is the γ 1 isoform of phospholipase C (PLCγ1) (Park, 1991, Weiss, 1991, Secrist, 1991, Nishibe, 1990). Tyrosine phosphorylation of PLC gamma 1 increases its catalytic activity leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (Nishibe, 1990, Kim, 1991). These second messengers, in turn, induce the mobilization of intracellular calcium $Ca^{++}$ and the activation of protein kinase C (PKC) respectively (Figure 3) (Berridge, 1984, Imboden, 1985).

Figure 3

Distal signal transduction pathway in T cells. Note that two distinct pathways are activated. The $Ca^{++}$/calcineurin path leads to activation of NF-AT induced transcription, and the ras/MAP kinase path leads to Fos/Jun induced transcription


PTK
p-PLCγI
PIP2
DAG
IP3
increased [Ca2+]
PKC
ras
calcineurin
MAP kinase
NF-ATc
NF-ATn
IL-2 gene

Interactions of PTK in TCR Function

Whereas ZAP 70 and the Src-family PTKs are structurally and functionally distinct, these two classes of PTKs participate and interact in the initial events involved in TCR signal transduction function. The efficient association of phosphorylated ZAP-70 with phosphorylated ζ requires the presence of Lck (Iwashima, 1993). Lck is responsible for initiating phosphorylation of the TCR ζ chain and subsequent phosphorylation of ZAP-70 itself (Labadia, 1996).

Lck associates with the TCR cytoplasmic domains and initiates phosphorylation of the ARAMs of the CD3 and ζ subunits, which in turn recruit ZAP-70 to this activated receptor complex. The association of ZAP-70 with phosphorylated ζ is mediated by SH2-phosphotyrosine interactions. Both SH2 domains are required for the efficient and stable association of ZAP 70 with ζ. Both tyrosine residues within each ARAM must be phosphorylated for its efficient and stable interaction with ZAP-70. Each of the two tandemly arranged SH2 domains of ZAP-70 bind one of the two phosphorylated tyrosine residues within each ζ ARAM motif. The association of ZAP-70 with the phosphorylated ζ ARAM sequences recruits ZAP-70 to the activated receptor complex and results in tyrosine phosphorylation of ZAP 70 by Lck (Figure 2). The kinase activity of Lck is required but the association of ZAP 70 with phosphorylated ζ is independent of the kinase activity of ZAP-70 (Iwashima, 1993).

The CD3 ε subunit has also been implicated in TCR signaling. Recent studies have demonstrated that the CD3 ε subunit undergoes tyrosine phosphorylation and associates with the phosphorylated form of ZAP 70 upon TCR stimulation (Straus, 1993, Qian,

1993). The fate of developing CD4 CD8 thymocytes is determined by signals transduced through surface TCR complexes. It was reported that cross-linking of TCR on CD4 CD8 thymocytes fails to activate ZAP-70 and fails to initiate downstream signaling events, unless the TCR are coaggregated with surface coreceptor molecules (Wiest 1996)

In one study, investigators reconstituted Lck phosphorylation of TCR ζ and ZAP-70 recruitment in heterologous cells and examined the role of the tyrosine phosphatase HCP in regulating the process. Both Lck and Fyn induce significant phosphorylation of TCR ζ. However, under conditions of comparable Lck and Fyn expression, Lck was found to induce 3-to 4- fold greater in vivo phosphorylation of TCR ζ. HCP dephosphorylated Lck, ZAP-70 and the TCR ζ chain. Dephosphorylation of the different TCR ζ isoforms results in disruption of the interaction between TCR ζ and ZAP 70. These results indicate that HCP acts to negatively regulate signal transduction pathways in T cells (Raab, 1996)

The protein-tyrosine kinase Csk is one of the main down regulators of the Src family of kinases. Csk may be involved in the down regulation of TCR signaling by C-terminal tyrosine phosphorylation of Lck and Fyn, however it is not known how Csk activity is regulated or how it targets these Src family members. Data demonstrate that the SH2 domain of Csk specifically associates with at least two tyrosine-phosphorylated proteins in normal human T cells and that this association is independent of TCR/CD3 activation, and that Csk may be a part of a multiprotein complex containing GAP (Catipovic, 1996)

## The Role of Calcium in T Cell Activation

### Calcium Ion ( $Ca^{++}$ )

Release of intracellular calcium from intracellular stores and influx from the extracellular medium is one of the hallmarks of cellular activation in many different cell types. Upon stimulation of normal T cell populations with specific and nonspecific mitogens, intracellular free calcium ion levels rise dramatically. Both $Ca^{++}$ signals and the activity of PTKs are essential for the optimal expression of c-jun in response to TCR/CD3 signals (Liu, 1996). Tyrosine kinases indirectly raise intracellular calcium concentration by activating phospholipases (PL-C) that generate inositol 1,4,5 trisphosphate (IP3) (Figure 3). IP3 activates the IP3 receptor (IP3R), an intracellular calcium release channel on the endoplasmic reticulum. T cell receptor stimulation triggers a physical association between Fyn and the IP3R, which induces tyrosine phosphorylation of the IP3R. Fyn activates an IP3-gated calcium channel in vitro, and tyrosine phosphorylation of the IP3R during T cell activation is reduced in thymocytes from Fyn -/- mice (Jayaraman, 1996).

Activation through the $Ca^{++}$/calcineurin pathway is essential to the transcription of many cytokine genes. The conserved cis-acting sequence GGAAAA, and transcription factors binding to this sequence are involved in the response to increased intracellular $Ca^{++}$ concentrations. The identification and importance of the same sequence in a non-cytokine gene, the human γ TCR chain enhancer was reported (Hettmann, 1995).

By using α/β TCR mutant thymus cultures, one study showed that direct stimulation of intracellular signals by PMA and the calcium ionophore ionomycin can induce the generation of mature CD4 $^{+}$ T cells, bypassing TCR-induced positive selection

signals. The concentrations of phorbol ester that induced positive selection were more restricted than those that induced mature T cell activation. The combination of phorbol ester and ionomycin restored the generation of CD4⁺8⁻ T cells in class II MHC⁻ thymus cultures, but did not induce the generation of CD4⁻8⁻ T cells in class I MHC⁻ thymus cultures. These results identify that the combination of protein kinase C activation and calcium elevation is the biochemical signal that can induce positive selection of CD4⁺ T cells (Takahama, 1996).

Calcineurin

Calcineurin (CaN) is a calcium- and calmodulin-dependent protein serine/threonine phosphatase which is critical for several important cellular processes, including T cell activation. Increases of intracellular free calcium result in the activation of this phosphatase (Kissinger, 1995). One function of calcineurin is to dephosphorylate the cytoplasmic component of NF-AT which allows it to translocate to the nucleus and stimulate transcription of the IL-2 gene among others. Calcineurin also synergizes with PKC-induced activation of nuclear factor (NF)-kappa B in T cell lines (Steffan, 1995).

# CHAPTER 3
# INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

Intraepithelial lymphocytes of the intestinal epithelium (iIEL) represent a phenotypically and functionally distinct subpopulation of peripheral T cells. iIEL have an unusual surface phenotype and response to stimulation when compared to peripheral blood lymphocytes and splenic lymphocytes

## Phenotype

### General

iIEL consist of a population of mainly CD3 T cells, approximately 50% of which express the γδ TCR and 50% express the αβ TCR. iIEL are predominantly CD8 but, in contrast to peripheral or circulating CD8 T cells, which are all Thy 1 , a subset of the CD8 iIEL is Thy-1 Experiments show that CD8 iIEL consist of two ontogenically distinct populations, one bearing CD8 molecules made of two different polypeptide chains (Lyt-2 or α, and Lyt-3 or β chains), and the other bearing CD8 molecules lacking β chains These two populations are named heterodimeric CD8α/β and homodimeric CD8α/α In CD8α knockout mice, the population size of iIEL remained normal but the majority of iIEL were CD4 CD8 T cells expressing αβ or γδ T cell receptors (Fung-Leung, 1994) CD8β gene-targeted mice were generated in a study of CD8 function in the intestine. The CD8α⁺ T cell population in the thymus and in most peripheral lymphoid organs was reduced to 20-30% of that in wild type littermates CD8α expression on

thymocytes and peripheral T cells also decreased to 44 and 53% of the normal levels respectively. Neither the population size nor the CD8α expression level of CD8α/α iIEL was reduced (Fuegg-Leung, 1994).

Repertoire Development

The study of repertoire development throughout the length of the small intestine has revealed that some clones are present all along the epithelium, suggesting that the CD8 α/α iIEL and CD8 α/β iIEL are capable of recirculating or migrating in the intestine. It has been proposed that the repertoire of αβ TCR iIEL is derived from a restricted number of T cells that either develop in situ or that arrive through circulation and can then undergo clonal expansion (Regnault, 1994). Within any given mouse, the repertoires expressed in αβ TCR, both CD8 α/α and CD8 α/β iIEL populations are oligoclonal and nonoverlapping between the two subsets. The clones are largely conserved through the length of the small intestine of the same individual (Regnault, 1994). TCR δ gene rearrangements in iIEL were studied in athymic radiation chimeras using polymerase chain reaction (PCR) and sequence analysis of DNAs spanning the variable (V), diversity (D), and junctional (J) genes. Evaluation of PCR amplified cDNAs made from iIEL RNA indicated that all four rearranged Vδ genes were expressed in iIEL from athymic mice (Stuckney, 1993). Other results show that Vγ1-bearing lymphocytes account for the largest population of γδ T cells in the mouse. This population includes a thymus-dependent component that homes to the secondary lymphoid organs and the purported thymus-independent component that constitutes a major fraction of the γδ iIEL (Ferrarini, 1994).

TAP and Beta-2 microglobulin

Beta-2 microglobulin (β2m) is a component of MHC class I and TAP is an assembly factor for this molecule and presented antigen. Recent data suggests that TAP-independent but β2m-requiring nonclassical class I molecules expressed by cells in the intestine, such as the thymus leukemia antigen (TLA) and CD1, could play a pivotal role in the development and/or the accumulation of major subpopulations of αβ TCR iIEL (Sydora,1993a). These and other results indicate that the generation of CD8 α/α lymphocyte population of αβ and γδ TCR iIEL is not dependent, but that of CD8 α/β lymphocyte population of αβ TCR iIEL is dependent on β2m and/or TAP1-dependent MHC class I molecules, expressed by the controlling cells present in the anatomical site, where the development of iIEL takes place (Fujiura, 1994). Other researchers concluded that the absence of β2m renders γδ TCR iIEL resistant to TCR-mediated inactivation and promotes activation of αβ TCR, CD8 α/α iIEL. The activation of γδ TCR iIEL seems to be directly controlled by β2m/MHC class I expression and independent from αβ TCR, CD8 α/β iIEL. Regulation of self reactive thymus-independent iIEL through β2m/MHC class I may contribute to control of autoreactive immune responses in the intestine (Neuhaus, 1995).

Hormone Effect on iIEL

Administration of the neuropeptide thyrotropin-releasing hormone (TRH) to adult mice thymectomized as neonates leads to the development of all iIEL T cells. Thus, a neuroendocrine signal initiated by the thymus during fetal or neonatal life appears to be required for subsequent extrathymic maturation of gut αβ TCR T cells (Wang,1994). In a

similar study, adult neonatal thymectomized (NTX) mice treated with exogenous thyroid stimulating hormone (TSH) for 3 weeks developed normal proportions and numbers of thymus dependent (TD) αβ TCR, CD8 α/β , CD5 , and Thy-1 iIEL in a manner similar to that of TRH-treated NTX mice The effect of hormone therapy was long-lasting in that iIEL subsets which developed during TSH treatment were present well after the known lifespan of TSH in vivo, and because the numbers of iIEL remained higher than in untreated mice These findings suggest that TD iIEL develop extrathymically through a series of immunologic and hormonal interactions which are regulated by the thymus during some phase of embryonic life (Wang, 1995)

In one set of experiments most of the αβ TCR iIEL whose development was inhibited by cyclosporin A (CSA, blocks T cell responses in vitro by inhibiting the phosphatase activity of calcineurin, preventing the activation of cytokine gene transcription) belonged to the CD4 CD8 α/α subset The development of extrathymically and thymically derived γδ TCR iIEL was completely resistant to CSA. The phenotype of CSA resistant γδ TCR iIEL in these models was not different from those in control mice, and the γδ TCR iIEL in CSA treated mice appear to be mature and activated as most were large, granular, and CD69 These results suggest that despite their similarity, the intracellular activation cascade involved after TCR stimulation between αβ TCR, CD4, CD8 α/α and γδ TCR, CD4, CD8 α/α iIEL are markedly different (Lin, 1994a)

CD Molecule Expression

Cluster designation (CD) molecules are expressed on the surface of cells and are used to phenotypically catagorize cells There are many different molecules with CD

numbers that have recently been identified. The lack of CD5, which is normally found on all peripheral, lymph node and spleen T cells, defines a unique subset of intraepithelial T cells expressing either γδ or αβ TCR that include potentially autoreactive cells that remain anergic in the absence of IL-2 ( Croitoru, 1994). Other studies emphasize the expression of CD7 by iIEL T cells and reveal the existence of an undefined structural difference between CD7 molecules on iIEL compared to thymocytes (Russell, 1994). The CD2 molecule is normally expressed on nearly all murine lymphocytes, and is costimulatory in T cell activation via the TCR. A naturally occurring T lymphocyte population that is bimodal for CD2 expression was found in iIEL. αβ TCR iIEL contain CD2$^-$ and CD2$^+$ cells of approximately equal proportion, while γδ TCR iIEL are predominantly CD2$^-$ (Van Houten, 1993). The CD28 antigen has been recently demonstrated to be a costimulatory molecule and is expressed by almost all thymic and peripheral αβ and γδ TCR cells in the mouse system. It was shown that expression of CD28 is heterogeneous among iIEL. Whereas some αβ TCR expressing iIEL subsets such as CD4$^+$8$^+$ and CD4$^-$CD8$^+$ α/β cells express CD28 at the same levels as their phenotypic counterparts in lymph node, other subsets of αβ TCR cells (including CD4$^-$, CD8α/α and CD4$^-$CD8$^-$ α/α cells) as well as γδ TCR iIEL fail to express CD28 (Ohteki, 1993). In another study, it was reported that the production of a mAb, designated HL3, exhibited reactivity with a subset of iIEL. In differential screening assays, HL3 reacted with 30-50% of iIEL, but not with T cells of the thymus, spleen, or lymph nodes. Biochemical characterization revealed that the HL3 mAb recognized p150,95 (CD11c/CD18, CR4), a member of the β2-integrin family

Fluorescence flow cytometric analyses showed that p150 95 was expressed by αβ TCR or γδ TCR, CD4 , CD8 iIEL but not by CD4 ,CD8 iIEL (Hiraatt 1995)

Effects of Age

Age-related changes in T cell subsets were examined In 2 month-old mice bred under conventional condition, iIEL consisted of a large number of CD4 , CD8α/α cells bearing either αβ TCR or γδ TCR and only a few CD4 CD8α cells In aged mice (6 months old and 24 months old) the CD4 CD8α/α iIEL bearing αβ TCR increased in number and conversely the proportion of γδ TCR iIEL was decreased Such an increase in number of CD4 , CD8 α/α cells was detected in iIEL from aged (14-months old) nude mice, but not in aged (14 months old) germ-free mice (Takeuchi, 1993)

Other Models

iIEL of calves were analyzed phenotypically and compared with peripheral blood lymphocytes (PBL) via flow cytometry using monoclonal antibodies to cell surface markers Approximately 25% of PBL and iIEL expressed the γδ T cell receptor There were approximately twice as many CD4 cells as CD8 cells in the PBL, whereas there were three to five times as many CD8 cells as CD4 cells in the iIEL These results indicate that iIEL of calves also are antigenically distinct from PBL (Waters, 1995) In another bovine study, leukocytes from the bovine intestinal epithelium were isolated and assessed for their ability to inhibit in vitro replication of an enteric pathogen, bovine coronavirus (BCV) iIEL were also tested for their ability to mediate different types of cytotoxicity The iIEL were able to inhibit virus replication, and this activity was markedly enhanced by interleukin-2 and tumor necrosis factor This combination of

cytokines has similar effects on iIEL mediated cytotoxicity which implicated cytotoxicity as a mechanism by which viral replication was limited (Godson, 1992)

In a study of wild mice the majority of iIEL were CD3 , CD8 CD4 T cells CD4 CD8 also were present in iIEL Isolates from wild mice, although at low numbers Among iIEL, but not T cells from the spleen or lymph nodes there was a notable lack of Thy-1 expression, a preponderance of CD8 α/α T cells and a relatively high ratio of γδ TCR T cells over αβ TCR T cells, suggesting that some iIEL in wild mice may develop via an extrathymic pathway similar to that described for laboratory mice (Mosley 1994b)

In chickens, postnatal development of various iIEL T lymphocyte subpopulations expressing CD3 CD8, CD4, and antigen-specific TCR heterodimers expressing γδ (TCR1) or αβ (TCR2) was investigated using specific monoclonal antibodies and flow cytometry iIEL expressing the CD8 antigen increased gradually following hatching and subsequently declined with age. The CD4 cells represented a minor subpopulation among the iIEL The ratio of iIEL T cells expressing γδ (TCR1) to αβ (TCR2) in the intraepithelium of chickens gradually increased after hatching (Lillehoj 1993) Also, the major T lymphocyte subpopulations expressing CD8, CD4, αβ TCR (TCR2) or γδ TCR (TCR1) were assessed in duodenal intraepithelium of two inbred chicken strains, SC and TK, by flow cytometry at various times after oral inoculation with *Eimeria acervulina* A significant increase in the duodenal CD8 iIEL occurred in the SC chickens which manifested as a lower level of oocyst production compared to the TK chickens following primary and challenge infections These results suggest that variations in T cell subpopulations may reflect eimerian infection-related changes in the gut-associated lymphoid tissues and that a significant increase in CD8 iIEL in SC chickens may reflect

enhanced acquired immune status in SC chickens compared to TK chickens (Lillehoj, 1994)

## Function

### Expansion and Colonization in vivo

iIEL particularly αβ TCR bearing iIEL, dramatically increase in number after microbial colonization of formerly germ free (ex-GF) mice (Umesaki, 1993) These and other results strongly suggest that the number of iIEL, particularly αβ TCR iIEL, increases after microbial colonization through recruitment into the cell cycle of a large proportion of iIEL or their immediate precursors The difference in the relative BrdUrd-labeling rate of αβ iIEL compared to that of γδ iIEL between germ-free and conventionalized mice indicates a preferential increase in the αβ IEL subset during the course of conventionalization (Imaoka, 1996) The observation that the αβ TCR iIEL populations comprise a few hundred clones of very heterogeneous size and distribution suggests that they arise from a limited number of precursors which may be slowly but continuously renewed, and undergo extensive clonal expansion in the epithelium (Regnault, 1994) αβ TCR and γδ TCR iIEL show different rates of division in different sections of the gut, perhaps reflecting responses to different antigens Both αβ TCR and γδ TCR iIEL reside in the epithelium for weeks during which time the gut epithelial population will have been renewed many times (Penney, 1995) Other results demonstrate a developmentally ordered expression pattern of the iIEL αE β 7 integrin that suggests a common function for this integrin during γδ and αβ TCR, CD4 , CD8 T cell thymocyte development or perhaps in effector functions for these subsets ( Lefrancois, 1994)

Proliferation and Cytokine Production

Most iIEL respond poorly to stimuli that have been shown to cause rapid proliferation and activation in other T cell populations. However, closer analysis of the various subpopulations of IEL reveals interesting differences between cells that express different surface markers and are thought to have different requirements for development.

In iIEL, staphylococcus enterotoxin E (SEE) induced no IL-2, IL-4, or gamma interferon but produced IL-6 and TNF, whereas staphylococcus enterotoxin B (SEB) stimulation resulted in no IL-2 or gamma interferon but did result in detectable IL-4, IL-6, and TNF (Sperber, 1995). Two-color flow cytometric analysis showed that in vitro stimulation of iIEL with SEB resulted in the expansion of the thymus dependent but not the thymus independent iIEL; the CD8 α/α iIEL were functionally non-responsive to stimulation with SEB (Siebrecht, 1993). Thy-1-enriched and Thy-1 depleted lymphocytes from murine intestinal epithelium were studied separately for their ability to proliferate and secrete lymphokines in vitro after stimulation with SEB. Thy-1-enriched iIEL proliferate and secrete lymphokines after stimulation with SEB. The majority of Thy-1-depleted iIEL do not proliferate, and secrete only minimal levels of lymphokine to any of the stimuli tested in this study (Viney, 1992).

CD8+ lymph node (LN) cells require both TCR and CD28 triggering for activation. However, CD8 α/β iIEL proliferate and produce IL-2 and IFN-gamma when stimulated with anti TCR β mAb alone. On the other hand, CD8α/α iIEL neither make IL-2 or IFN-γ nor proliferate even when both stimuli are provided (Gelfanov, 1995). Human αβ TCR+ iIEL could not be driven to proliferate or exhibit cytotoxic function in vitro, however, PCR analysis for detection of lymphokine mRNA revealed constitutive production of

several lymphokines known to exert trophic effects on intestinal epithelial cells and pro-inflammatory activities (Plusehke 1994)

iIEL proliferate weakly in response to CD3/TCR ligation However, ligation of the CD3/TCR complex could elicit a rapid cytotoxic response and serine esterase release by iIEL The unusual functional capabilities and the activation state of iIEL are independent of the TCR isotype expressed by these cells (Svdora, 1993b) A soluble extract of *Mycobacterium tuberculosis* (Mtb) but not purified protein derivative of tuberculin, induced significant proliferative responses in iIEL cultures The active component was apparently a heat shock protein (HSP), since recombinant 71-kDa HSP from Mtb induced iIEL to proliferate, while 65 kDa HSP from *M bovis* and *M leprae* did not Both αβ and γδ TCR-enriched iIEL gave proliferative responses to 71 kDa HSP Results show that the 71-kDa HSP of Mtb induces iIEL T cells to divide and to secrete cytokines (Beagley, 1993) The nature and cell cycle stage of cytokine-producing T lymphocytes in iIEL was determined by using IFN-γ– and IL-5-specific ELISPOT assay cytokine specific mRNA-cDNA dot-blot hybridization and PCR, and flow cytometry (FACS) for DNA analysis Results suggest that αβ TCR and γδ TCR iIEL can produce cytokines without cell proliferation (Yamamoto, 1993)

Signal Transduction

Because of the different results obtained from proliferation experiments and analyses of other cell surface receptor associated functions, there has recently been interest in the pathways through which signals travel from the surface of the cell to the nucleus, where transcription of genes that are responsible for these functions is initiated The MAP kinase family of signal transduction molecules translocate from the cytoplasm of the cell to

the nucleus and have been implicated in the response of many kinds of cells to a variety of stimuli. These and other signal transduction molecules and pathways will be discussed in the following section.

Freshly isolated iIEL have a high intracellular microtubule-associated protein kinase-2 (mitogen activated protein) (MAP 2K) activity level, suggesting that these cells are activated despite their weak proliferative response. Consistent with this, MAP 2K is tyrosine-phosphorylated in both untreated and PMA-treated iIEL is elevated. MAP 2K activity also is elevated in iIEL from germ-free mice, demonstrating that activation does not depend on normal levels of exposure to bacterial flora. CD8 α/α iIEL, whether bearing αβ or γδ TCR, contain, in normal mice, mRNAs for both ζ and Fc epsilon RI γ chains. These chains are present in their CD3-TCR complexes as homo- or heterodimers. In contrast, only ζ chain mRNA and homodimers are found in gut CD8 α/β iIEL and in peripheral T lymphocytes. Use of different TCR transducing modules and CD8 accessory molecules between the thymus-independent and the thymus-derived T cell populations provides one possible explanation for their difference in reactivity to antigenic stimulations (Guy Grand, 1994)

Cytotoxicity

iIEL have been shown to be cytotoxic in culture without prior priming by antigen or antigen presenting cells. This is in contrast to all other CD8⁺ lymphocytes, including peripheral blood and splenic T cells, that need to be primed with antigen or IL-2 to exert cytolytic effector function. One way of investigating this phenomenon is to use cell lines established from iIEL

Three T cell lines, isolated from murine small intestine epithelia, have been studied with respect to phenotypic properties and cytotoxic activity. All lines were αβ TCR+, Thy-1+, CD3+, CD4-, CD8+ but differed in that one line was CD8 α/α, CD5-, one line was CD8 α/α, CD5+, and one line was CD8 α/β+ CD5+. Both the CD8 α/α, CD5- and the CD8 α/β, CD5+ lines lysed antigen-bearing target cells; however, the latter line also spontaneously lysed natural killer (NK)-sensitive target cells. The CD8 α/α, CD5- iIEL line was nonlytic for antigen-bearing target cells, for NK-sensitive target cells, and in assays that detect lytic activity regardless of specificity. These findings demonstrate that cytotoxicity of murine iIEL resides among multiple phenotypic subsets (Mosley, 1994a). Two other cell lines were established from mouse iIEL, Thy-1+ CD5+ αβ TCR, CD4- CD8 α/α (double-positive, DP) iIEL and Thy-1+ CD5+ αβ TCR+ CD4- CD8 α/α (CD8 single-positive, CD8 SP) iIEL. The CD8 SP iIEL cell line had cytotoxic activities and was triggered to proliferate by TCR-directed stimuli. The DP iIEL cell line expressed high levels of the αβ TCR, exhibited cytotoxic activity in redirected lysis assays, and had perforin in the cytoplasm, indicating the functional maturity of this cell line. However, the DP iIEL cell line did not proliferate in response to αβ TCR-directed stimuli, which indicated that αβ TCR-mediated signalling was able to initiate cytotoxic function but not to induce proliferation of the DP iIEL cell line. The unique properties of the DP iIEL cell line seems to support the notion that DP iIEL may undergo a unique maturation process in the gut microenvironment (Sasahara, 1994).

When lymph node (LN)-T blasts (greater than 75% CD4+) activated with anti-CD3 were treated with CD8-depleted iIEL, intact cellular DNA of the T blasts disappeared

within 1 h with increased amounts of small-sized DNA. These results suggest that CD8-iIEL directly and nonspecifically kill lymph node CD4 T blasts and possibly down regulate TCR-mediated proliferation of peripheral T cells in the gut epithelium (Kawamoto 1996)

Recent results indicate that most of the iIEL subpopulations develop independently of passage through PPs and mesenteric LNs and that the *aly* mutation, which induces a generalized lack of lymph nodes (LNs) and Peyer's patches (PPs) accompanied by immunodeficiency in mice, interrupts cytotoxic iIEL development during relatively late differentiation steps that convert cytotoxic precursors to the constitutively cytolytic state (Nanno 1994) Human iIEL demonstrate target cell restricted spontaneous cytotoxic (SC) activity that is due to CD2 , CD3 , CD8 CD16⁻, CD56 effector cells, they kill epithelial cell (EC) tumours (such as DLD-1 colon cancer cells), but not NK-sensitive K-562 cells The susceptibilities of DLD-1 cell clones to lysis by iIEL and PBL effector cells did not correlate, suggesting different mechanisms of lysis Antibody blocking experiments showed that the main surface molecules involved in lysis depended on the effector cell type αEβ7 (HML-1) on iIEL and CD16 on PBL (Roberts, 1993b) Others have analyzed the cytolytic activity of freshly isolated iIEL of several different mouse strains in an anti TCR monoclonal antibody mediated redirected lysis assay The cytolytic activity of γδ iIEL but not that of αβ iIEL was strain-dependent (Ishikawa, 1993)

Development

Many recent results indicate that certain subpopulations of IEL may develop independently of the thymus as opposed to other populations of T cells such as peripheral blood, splenic and lymph node T cells While some of the data are conflicting it is

generally agreed that at least the Thy 1 , CD3 , CD8α/α, γδ TCR iIEL develop independently of intact thymus tissue.

Results from experiments in which RAG-/- mutant mice, which have no T cells, were injected either with the bone marrow (BM) cells of nude mice or with selected peripheral lymph node (LN) T cells of euthymic mice demonstrate that the thymus and/or thymic-derived peripheral T cells are absolutely required for the generation of CD4 , CD8 α/β and CD4 CD8 α/α iIEL, which are thus thymus-dependent In contrast, TCR CD8 α/α iIEL appear in the absence of the thymus, and may be thymus independent (Rocha, 1994) It has also been shown that CD3 CD8 CD3 CD8 α/α and CD3 CD8 α/α iIEL, respectively, appear sequentially in normal ontogeny and in bone marrow-reconstituted thymectomized radiation chimeras In the latter, virtually all CD3 CD8 α/α iIEL expressed the γδ TCR, but not the αβ TCR. From this and what is presently known about T cell development, it was proposed that CD3 , CD8 iIEL are an intermediate in extrathymic iIEL development and that the development of extrathymically derived iIEL occurs at the intestinal epithelium from CD3 CD8 to CD3 , CD8 to CD3 (γδ TCR) CD8αα (Lan, 1994b) iIEL injected into SCID mice preferentially repopulate the intestinal epithelium Tha recovered cells are of donor-type origin and exhibit a typical iIEL phenotype The donor type T lymphocytes that can sometimes be found in other organs of iIEL-repopulated SCID mice are generally of low number They are not stained with antibodies against iIEL-specific markers and their phenotypes appear to be more typical for T cells normally found in these sites In contrast, the intestinal epithelium of SCID mice cannot be efficiently repopulated with lymphocytes using cells of other organs including thymocytes, Peyer's patch lymphocytes, and bone marrow cells

From these data it was concluded that intestinal iIEL are confined to the intestinal epithelium and possibly contain a precursor-type cell that preferentially regenerates cells of its own population (Sydora, 1993a)

Congenitally athymic nude mice have low numbers of γδ TCR iIEL as well as very few αβ TCR iIEL far less than would be expected if one assumes that γδ TCR iIEL and αβ TCR (CD8 α/α and Thy-1⁻) iIEL in euthymic mice are extrathymically derived. To examine this discrepancy researchers followed extrathymic iIEL differentiation in iIEL of day 3-thymectomized (NTX) mice as another athymic mouse model and found that γδ TCR iIEL and extrathymically derived αβ TCR iIEL in NTX mice are markedly reduced, almost to the level of nude mice. It is probably the absence of a thymic stroma that is responsible for the lower amounts of extrathymically derived iIEL in nude mice as the low amounts can be corrected to euthymic levels by syngeneic fetal thymus grafting but not by direct injection of F1 thymocytes

Unlike TCR/CD3 extrathymically derived iIEL a large proportion of extrathymic CD3⁺, CD8⁺ and CD3⁻, CD8⁺ iIEL were threefold more frequent in nude and NTX than in euthymic mice This suggests that the thymus influences extrathymically derived iIEL in its development from CD3⁻ to CD3⁺ at the small intestinal epithelium (Lin, 1993) It has also been reported that the thymus is an integral component of iIEL maturation and is required for a novel two-stage process of T cell production In neonatally thymectomized mice γδ TCR iIEL were depleted and some αβ TCR iIEL were of an immature phenotype Thymus grafting experiments revealed that all γδ TCR and αβ TCR iIEL subsets could be thymus-derived, including αβ TCR cells lacking Thy1 and CD8 β In utero anti-γδ TCR mAb treatments resulted in depletion of γδ iIEL without subsequent re-emergence of this

subset in adulthood, whereas anti-αβ TCR mAb treatment only marginally reduced the αβ iIEL subset. These findings suggest that αβ TCR and γδ TCR iIEL arose at distinct developmental stages. The results indicate that some iIEL precursors are thymus-derived but require further thymic influence to mature in the periphery (Lefrancois, 1994).

Interactions between stem cell factor (SCF) and its receptor, c-Kit, are important for development of hematopoietic, melanocytes, and germ cells. T lymphocytes appeared normal in c-Kit or SCF mutant mice, except for the iIEL. In mutant mice, beginning at 6-8 weeks of age, the number of γδ iIEL decreased, whereas αβ iIEL increased. The latter was due largely to an increased CD4+, CD8+ αβ TCR subset, suggesting that these cells may be intermediates in the αβ iIEL lineage. c-Kit or SCF was expressed by iIEL or intestinal epithelial cells, respectively, indicating a potential for direct intercellular interaction. Thus, SCF-c-Kit interactions may be important for homeostasis of the intestinal immune compartment (Puddington, 1994).

Adhesion Molecule Expression

The differential expression of various adhesion molecules has been demonstrated in iIEL. These differences may, in part, explain the difference in reactivity of these cells to stimulation and their cytotoxic phenotype.

The expression of adhesion molecules by human duodenal iIEL was examined by two-color flow cytometry. Resting iIEL expressed LFA-1, HML-1 and CD44. Stimulation with phytohemagglutinin (PHA) resulted in down-regulation of expression of these molecules with induction of expression of ICAM-1 and VLA-4. Monoclonal antibodies to VLA-4, and to a lesser extent ICAM-1 blocked adherence of iIEL to I407 cells, a fetal intestinal cell line. These data suggest that VLA-4 may be involved in

adherence of iIEL to human enterocytes or secreted matrix molecules in vivo (Kelleher, 1994). The integrin α4β7 is expressed at high levels iIEL. It was reported that a mouse T cell hybridoma, MTC-1, with similar surface expression of this molecule, adhered strongly to cells of the mouse rectal carcinoma line CMT93 and that adhesion was blocked completely by the monoclonal antibody M290 (Roberts, 1993b). The mechanism by which iIEL bind to the colon cancer line, DLD-1, was evaluated. Binding of iIEL to DLD-1 was reduced by monoclonal antibodies to HML-1 and and was completely eliminated by antibodies to HML-1 and CD11a combined. Anti-HML-1 also inhibited the binding of iIEL to other epithelial cell (EC) tumors but did not affect binding to non-EC tumors or fibroblasts. The authors concluded that the binding of iIEL to EC tumors is mediated by HML-1 and CD11a (Roberts, 1993b). PHA and TGF-β stimulated iIEL hybridoma cells, but not control BW5147 cells (α4/β7 integrin negative fusion partner), bind to IEC-18 and CMT-93 intestinal epithelial cells, but not to renal mesangial cells. The binding was partially blocked by mAbs to integrin α4 and/or αM290 (Ni, 1995b).

# CHAPTER 4
# SPECIFIC AIMS

1) Test the hypothesis that there is evolutionary conservation of function and phenotype between chicken and mouse iIEL

Before the chicken can be used as model for study of iIEL, it must first be demonstrated that chicken iIEL are similar to the mouse model. To accomplish this, the phenotype and functional status of chicken iIEL must be determined. Flow cytometry will be used to determine the surface marker phenotype and proliferation assays in response to well characterized mitogens will be employed. Calcium release assays, a measure of the ability of a cell population to respond to calcium ionophores and a measure of activation, will also be used

2) Test the hypothesis that exogenous antigen overstimulation is not the reason for the refractory response of iIEL.

Use of embryonic iIEL from 20 day old fertile eggs will allow the study of iIEL that have never been exposed to exogenous antigen. By performing functional studies of these cells the question of whether overstimulation by exogenous antigens, food and microbial, that are encountered by these cells can be answered

3) Test the hypothesis that there are alterations in the signal transduction pathways between iIEL and splenic lymphocytes.

Because tIEL recognize antigens via their TCR in the same fashion as other T cells, yet fail to respond in the same fashion, the question arises whether tIEL transduce the message from the TCR to the nucleus in the same fashion as other cells or if their signal transduction pathways are altered either in the way that certain molecules behave or in the expression of different signal transduction molecules. This will be studied using stimulated and unstimulated cells in western blot analyses using antiphosphotyrosine antibodies and antibodies to specific signal transduction molecules.

# CHAPTER 5
# MATERIALS AND METHODS

## Animals

Fertile eggs for the Hyline White Leghorn line SC were obtained from Hyline International (Dallas Center, IA). Chickens were hatched and raised at the University of Florida Dept. of Dairy and Poultry Sciences. The chickens used for experiments were 2-5 months old. BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA) or from the Dept. of Pathology, Immunology & Laboratory Medicine breeding colony, and were used at age 6-12 weeks.

## Reagents

Fluo-3 and pluronic acid were purchased from Molecular Probes, Inc. (Eugene, OR). Ionomycin, A23187, EGTA, peroxidase-conjugated ExtrAvidin, ExtrAvidin, and $^3$H-thymidine were obtained from Sigma Chemical Co. (St. Louis, MO). Biotin-conjugated anti-phosphotyrosine mAb clone PY20 was from Calbiochem (La Jolla, CA), and reagents for enhanced chemiluminescent detection with western blots were from Amersham (Buckinghamshire, U.K.). Antibodies to chicken CD3, CD4, CD8, TCR1, TCR2 and TCR3 were obtained from Southern Biotechnology Associates (Birmingham, AL). The anti-αβTCR, anti γδ TCR, anti-CD3, anti MAP 2 kinase (which crossreacts with ERK-1 and ERK-2), anti-hamster IgG polyclonal, rabbit anti mouse IgG peroxidase conjugate and the goat anti-rabbit peroxidase conjugate were purchased from

Pharmingen Inc (San Diego, CA) The anti ZAP 70 antibody was purchased from Upstate Biological Inc (Lake Placid NY)

## iIEL Isolation

After cervical dislocation of mice or 20 day old embryos and $CO_2$ euthanasia of chickens small intestines were removed, opened longitudinally and washed 4 times with PBS without $Ca^{+}$ and $Mg^{+}$ and Peyer's patches were excised The intestines were then cut into 1-2 cm segments and incubated for 1 hour in 500 ml PBS plus 1 mM DTT and 1 mM EDTA at 37° C with stirring This slurry containing epithelial cells iIEL and intestinal tissue was passed over nylon wool columns 4 times using a new column for each pass The nylon wool columns were made from 60 cc syringes packed with 20 cc nylon wool and autoclaved After the fourth passage the eluate was collected in ten 50cc conical tubes (Sarstedt, Newton, NC) and centrifuged for 10 min at 300 x g. The pellets were resuspended in 35 ml 40% Percoll (Pharmacia, Piscataway NJ) and layered over 15 ml 70% Percoll in a 50 ml conical tube. This was centrifuged at 900 x g for 45 min and the interface containing 90-95% iIEL was harvested These were washed in PBS and resuspended in PBS at 2 x $10^7$/ml for further analysis.

## Proliferation Assays

Freshly isolated cells from chicken duodenum, ileum or mouse small intestine and chicken and mouse spleen were cultured in triplicate wells at a density of $2 \times 10^5$ cells/well in round-bottomed microtiter plates (Costar, Cambridge, MA) at 41°C in DMEM supplemented with 10% FBS, 2% chicken serum, 100 U/ml penicillin, and 100 U/ml streptomycin for chicken cells. For mouse cells the same conditions were used except that

the incubations were performed at 37°C and no chicken serum was added to the media Ionomycin and PMA were added to appropriate wells For CD3 crosslinking, plates were preincubated overnight at 4°C with 50 µl/well of anti-CD3 mAb (50 µg/ml in PBS), followed by washing with PBS As a control lymphocytes were cultured with medium alone. Cells were incubated for 3 days and $^3$H thymidine (0 5 µCi/well) was added 18 hr prior to harvesting Cells were harvested on a Micromate 196 cell harvester (Packard Meriden, CT) and thymidine incorporation was measured with a Matrix 96 direct β-counter (Packard, Meriden, CT)

Intracellular Calcium Measurement

For assessment of the relative levels of intracellular $Ca^{++}$ using fluo-3 cells ($1 \times 10^7$/ml) were incubated with 100 µg of pluronic acid F 127, and then loaded with 1 µM Fluo-3 in subdued light for 30 min at 25°C Cells were then washed thoroughly with HEPES buffered PBS and resuspended in 10 ml of Krebs ringer solution (125 mM NaCl, 5 mM KCl, 1 2 mM $KH_2PO_4$, 1 2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 0 05 mM diethylenetriamine pentaacetic acid and 25 mM HEPES-NaOH, pH 7 4) Fluo-3 loading was confirmed by lysing the cells with an EDTA-free lysis buffer and measuring fluorescence emission by the supernatant on a Perkin-Elmer cytofluorometer before and after addition of $CaCl_2$. Flow cytometric analysis ($1 \times 10^6$ cells/ml) of fluorescence was performed at 37°C for mouse lymphocytes and at 42°C for chicken lymphocytes with a FACSort (Becton-Dickinson Immunological Systems, San Jose, CA) using 488 nm and 530 nm wavelength for excitation and emission, respectively All of the measurements were completed within 2-3 hours after fluo-3 loading. Using the LYSIS II software

program, data of 4 parameters were collected in linear mode: forward light scatter (FSC), side light scatter (SSC) green fluorescence (FL1) and time. Viable lymphocytes were gated based on forward and side light scatter dot plots to exclude other cell populations and fragments. The fluo 3-loaded cells were run for 1 min to establish the baseline The calcium ionophore ionomycin or A23187 was then added to a final concentration of 1 μg/ml or 10 μg/ml, respectively, to induce the maximal intracellular $Ca^{++}$ concentration. In order to assess calcium mobilization from internal stores EGTA was added to a final concentration of 3 mM 2 min before the stimulus to chelate extracellular $Ca^{++}$ The time interval for collecting events was set at 500 ms per channel for a total time course of approximately 8 min Viabilities of remaining cells at the end of the experiments were >90%

Western Immunoblot Analysis

Cells were washed and resuspended in PBS at a concentration of $2 \times 10^7$ cells/ml 100 μl aliquots of cells were placed in microfuge tubes and incubated at 4°C for 1 hr with either anti-γδ TCR anti-αβ TCR mAb anti-CD3 mAb or with no mAb The cells were then washed with 1 ml ice cold PBS Cells were incubated for 5 min at 37°C for 5 min before addition of rabbit anti-mouse Ig as a crosslinking agent Cells were then incubated for 1, 5 or 10 min at 37°C After completion of stimulation, cells were washed with 1 ml of ice cold PBS and 25 μl of cold lysis buffer (50 mM Tris-HCl, pH 7 4, 100 mM NaCl, 1% sodium deoxycholate, 4% Nonidet P-40, 0 4% SDS, 2 mM PMSF, 100 μg/ml aproptin, 100 μM leupeptin, 1 mM $Na_3VO_4$) was added to stop the reaction. The tubes were placed on ice for 15 min and centrifuged for 10 min at 10 000 x G in a

microcentrifuge at 4° C Supernatants (20µl) were removed and 4 µl of 6x sample buffer (200 mM Tris-HCl, pH 6 8, 30% glycerol 6 6% SDS 16% 2 mercaptoethanol, 0 04% bromophenol blue) was added to the supernatants Samples were boiled for 5 min prior to loading. 20 µl of each sample were subjected to electrophoresis through a 10% SDS-PAGE gel (Bio-Rad Richmond, CA) and proteins were electrophoretically transferred to Immobilon-P membranes in a Bio-Rad semi-dry system

For detection of tyrosine-phosphorylated proteins, membranes were incubated for 1 hr with biotinylated anti phosphotyrosine mAb in reaction buffer (100mM Tris-Cl 0 9% pH 7 5 NaCl) The blots were washed three times in washing buffer (100 mM NaCl 50 mM Tris-HCl pH 8 0, 0 5 % Tween 20), and then incubated with peroxidase-conjugated ExtrAvidin (diluted 1 3,000 in reaction buffer) for 1 hr at room temperature The blots were washed three times in washing buffer and phosphotyrosine-containing proteins were detected by chemiluminescence by incubation with ECL western blotting peroxidase substrate (Amersham, Buckinghamshire, UK) according to the manufacturer s instructions and exposed to X-ray film. (Jersey Lab Supply, Viney, New Jersey)

For detection of specific signal transduction molecules membranes were incubated with anti-ZAP-70 or anti MAP 2 kinase or anti-Fyn antibody at a 1 1000 dilution for 1 hour at room temperature. These were then washed with wash buffer three times for 10 minutes each time The membranes were than incubated with the appropriate anti-IgG peroxidase conjugate at a 1 2000 dilution for 1 hour at room temperature Specific signal transduction molecules were detected by chemiluminescence by incubation with ECL western blotting peroxidase substrate (Amersham, Buckinghamshire, UK) according to

the manufacturer's instructions and exposed to X-ray film. (Jersey Lab Supply, Vineyard, New Jersey)

Granzyme B Assays

1 x $10^6$ freshly isolated iIEL and spleen cells were lysed in 0.1 ml of lysis buffer (1% Triton X-100, 50 mM Tris pH 8.0, 0.15 M NaCl), incubated on ice for 20 minutes and centrifuged at 10,000 x g for 10 minutes at 4° C. These lysates were stored at -70° a until ready for analysis. 20 µl of each sample was aliquoted into 3 wells of a 96 well ELISA plate and 180 µl of assay buffer (0.1M HEPES pH 7.0, 0.3 M NaCl, 1.0 mM EDTA, 0.11mM DTNB and 0.1 mM substrate, Enzyme Systems products, Dublin,CA) was added to each well. The plates were incubated at room temperature in the dark for 30 minutes and the optical density at 405 nm was measured. In some cases the cells were incubated for 48 hours with Con A or in wells that had been precoated with anti-CD3 before lysis. Optical density was plotted as a mean of the three wells per sample.

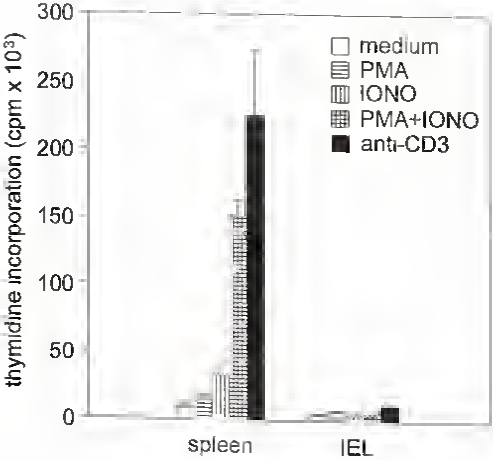
# CHAPTER 6
# RESULTS

## Chicken iIEL do not Proliferate in Response to Mitogens.

The chicken animal model was examined for its suitability for research on iIEL function because of several advantages to using chickens in this type of research 1) They are much larger than mice and offer a much higher yield of iIEL per animal 2) They are less expensive to purchase and maintain 3) Research on embryonic development is much more feasible The proliferative ability and phenotype of chicken iIEL were therefore compared to the mouse model which is well documented (Mosley, 1992) The iIEL of mice do not proliferate in response to mitogenic stimulation and their phenotype is well defined The response of chicken iIEL and splenocytes to several well known mitogens was studied and flow cytometric analysis was performed on these cells

For proliferation assays, chicken iIEL and splenocytes were cultured for 48 hours with PMA alone, ionomycin alone, PMA plus ionomycin, and in wells that had been precoated with anti-CD3 $^{3}$H-thymidine was added to the wells and the cells were cultured for another 18 hours The cells were harvested and counted in an automated counter Figure 4 indicates that chicken splenocytes proliferated maximally in response to anti-CD3, reaching an average of 225,000 cpm, and also to PMA plus ionomycin where average counts reached 150,000 cpm There was little response to PMA alone or ionomycin alone In contrast, chicken iIEL did not proliferate in response to any of these

Table 1 Phenotypic analysis of chicken and mouse lymphocytes Flow cytometry was performed on chicken and mouse IEL and splenocytes as described in Materials and Methods


300
250
200
150
100
50
0
thymidine incorporation (cpm x 10³)
medium
PMA
IONO
PMA+IONO
anti-CD3
spleen
IEL

mutogens These data parallel that which has been demonstrated in mice (Mosley, 1992) This indicates that chickens may serve as a good animal model for the study of iIEL.

Phenotype of Chicken iIEL.

Lymphocytes were isolated from chicken spleen, duodenum and ileum. These cells were incubated with flourescent antibodies for 1 hour, washed 3 times and analyzed by flow cytometry. Figure 5 is a contour plot of the flow cytometry data obtained after the analysis of adult chicken duodenal lymphocytes Figure 6 is a contour plot of the flow cytometry data obtained after the analysis of adult chicken ileal lymphocytes Figure 7 is a contour plot of the flow cytometry data obtained after the analysis of adult chicken splenic lymphocytes These results indicate that the cells that were isolated are similar in composition to those which have been found by other investigators and are summarized in Table 1 These data also indicate that the chicken iIEL are very similar to the mouse iIEL in their phenotypic composition and that chicken iIEL are distinct from splenic lymphocytes as is the case of the mouse model Taken together these data indicate that the chicken may be a good model for the study of iIEL.

Granzyme B is Expressed at Higher Levels in Freshly Isolated iIEL than in PBLs.

One of the properties of mouse iIEL that has been demonstrated is that they are cytolytic in a redirected cytotoxicity assay (Sydora,1994) Therefore, the activation status of chicken iIEL was measured by examining the intracellular expression of enzymes responsible for the cytolytic effector mechanism, such as granzyme B Elevated intracellular levels of this enzyme have been associated with an activated state in mouse splenic lymphocytes (McElhaney, 1996)

Figure 4

$^{3}$H thymidine incorporation by adult chicken iIEL and splenocytes in response to stimulation with PMA alone, ionomycin alone , PMA plus ionomycin and anti-CD3
Data is in counts per minute

| | Adult Chicken | | | Mouse |
|---|---|---|---|---|
| Antibody | Duodenum | Ileum | Spleen | iIEL |
| TCR1 γ/δ | 32.2 % | 41.5 % | 16.8 % | 43% |
| TCR2 α/β | 34.6 % | 30.5 % | 41.8 % | N/A |
| TCR3 α/β | 19.9 % | 19.8 % | 26.3 % | N/A |
| TCR2+3 | 54.5% | 50.3% | 68.1% | 46% |
| CD3 | 88.0 % | 85.0 % | 88.7 % | 90% |
| CD4 | 9.5 % | 6.7 % | 36.3 % | 14% |
| CD8 | 71.0 % | 77.0 % | 53.4 % | 88% |

Figure 5

Flow cytometric analysis of chicken duodenal intraepithelial lymphocytes.
FITC labelled antibodies (anti-CD3, TCR1, CD4) expressed on the Y axis and PE labelled antibodies (anti-CD8 TCR2, TCR3) expressed on the X axis

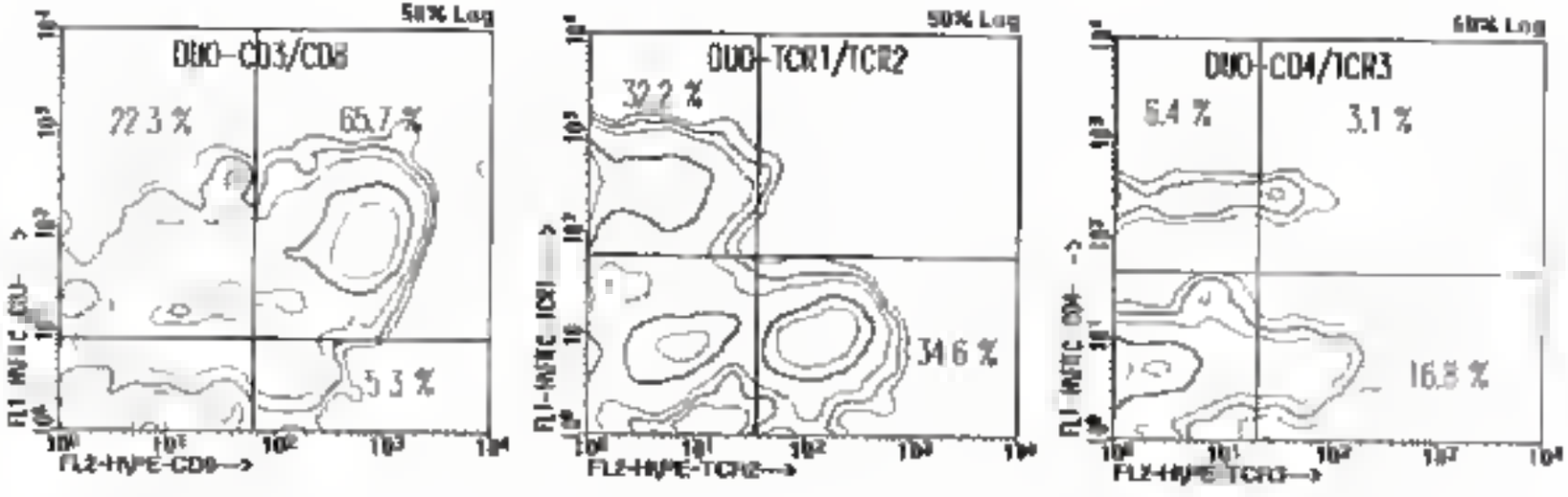

DUO-CD3/CD8
22.3 %
65.7 %
5.3 %
DUO-TCR1/TCR2
32.2 %
34.6 %
DUO-CD4/TCR3
6.4 %
3.1 %
16.8 %

Figure 6

Flow cytometric analysis of chicken ileal intraepithelial lymphocytes
FITC labelled antibodies (anti-CD3, TCR1, CD4) expressed on the Y axis and PE labelled antibodies (anti-CD8 TCR2, TCR3) expressed on the X axis.

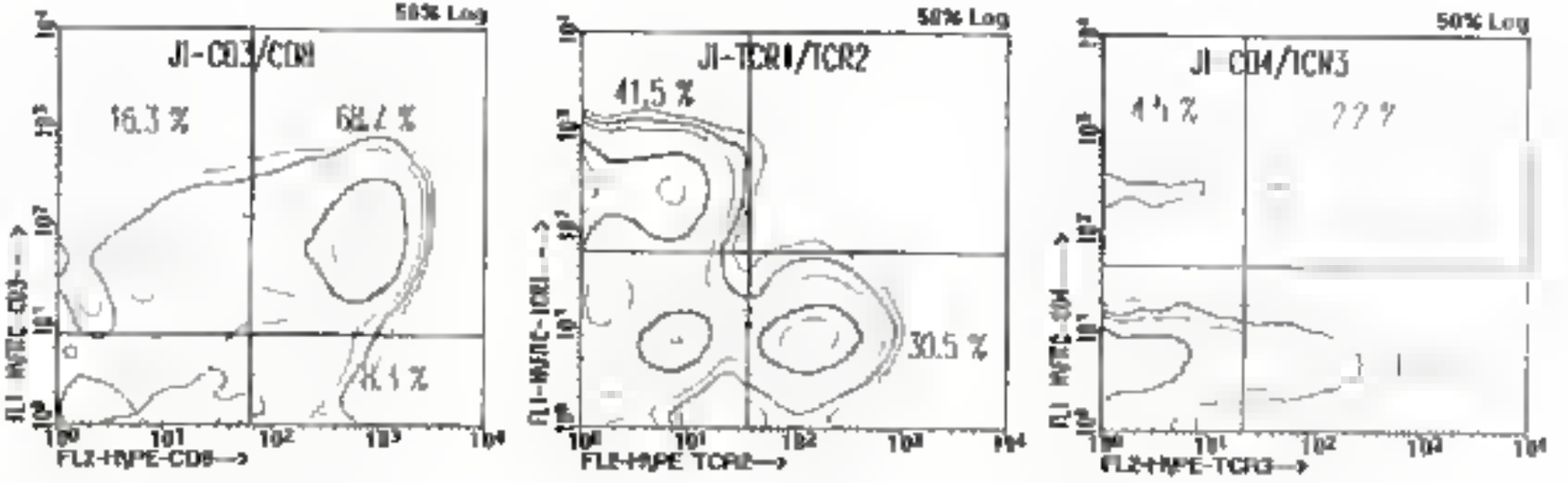
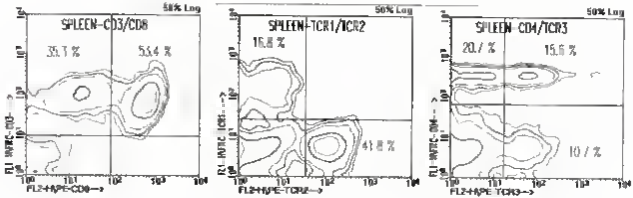

50% Log
Jl-CD3/CD8
16.3 %
68.7 %
FL1-HYPE-CD3—>
FL2-HYPE-CD8—>
50% Log
Jl-TCR1/TCR2
41.5 %
30.5 %
FL1-HYPE-TCR1—>
FL2-HYPE-TCR2—>
50% Log
Jl-CD4/CD3
FL1-HYPE-CD4—>
FL2-HYPE-TCRG—>

Figure 7

Flow cytometric analysis of chicken splenic lymphocytes. FITC labelled antibodies (anti-CD3, TCR1 γ/δ CD4) expressed on the Y axis and PE labelled antibodies (anti-CD8, TCR2 α/β, TCR3 α/β) expressed on the X axis.



50% Log
SPLEEN-CD3/CD8
35.3 %
53.4 %
FL1-HVRC-CD3-->
FL2-HVPE-CD8-->
50% Log
SPLEEN-TCR1/TCR2
16.8 %
41.8 %
FL1-HVRC-TCR1-->
FL2-HVPE-TCR2-->
50% Log
SPLEEN-CD4/TCR3
20.7 %
15.6 %
10.7 %
FL1-HVRC-CD4-->
FL2-HVPE-TCR3-->

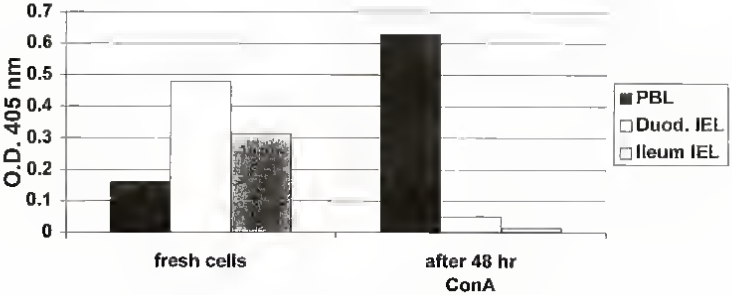
To evaluate the activation status of iIEL and assess their cytolytic potential, we measured intracellular granzyme B levels in stimulated and unstimulated chicken iIEL and PBL. iIEL and splenic lymphocytes were isolated from adult chickens. An aliquot of cells were lysed immediately and another aliquot were cultured for 48 hours with concanavalin A at 5 and 10 ug/ml and anti-CD3 antibodies then lysed. The intracellular granzyme B activity of each treatment group was assessed by incubating these supernatants with a granzyme B specific substrate coupled to a chromophore and analyzing optical density after 30 minutes. As seen in Figure 8, intracellular granzyme B levels were higher in iIEL immediately after isolation than they were in PBL, as indicated by an increase in the optical density of the reaction mixture after incubating cell lysates and a granzyme B specific substrate for thirty minutes. After stimulation for 48 hours with the mitogen Concanavalin A, which induces activation in PBL, the granzyme B levels in PBL was higher than when initially isolated and much higher than in iIEL. The viability of the iIEL was about 70% and PBL was about 90% at this time. This is consistent with the finding that iIEL are constitutively cytolytic in a redirected cytotoxicity assay (Sydora, 1993b) and demonstrate that some of the cytolytic effector mechanisms are differentially regulated in iIEL vs. PBL.

iIEL do not Mobilize Intracellular Calcium in Response to Calcium Ionophores.

One of the most universal features of a response to stimulation of a cell surface receptor is release of intracellular calcium. Calcium is an integral part of the signal transduction pathway that is stimulated upon engagement of the TCR. Calcium functions to activate calcineurin and other messenger molecules in the more distal portion of the

Figure 8

Intracellular granzyme B levels of IELs and PBLs before and after culture with Con A
Values expressed as optical density as described in materials and methods

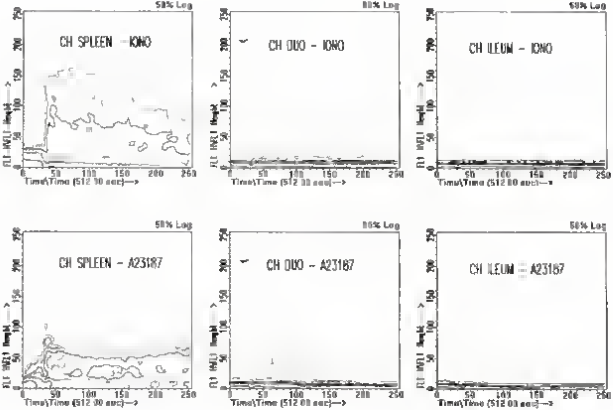

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. 405 nm
PBL
Duod. IEL
Ileum IEL
fresh cells
after 48 hr
ConA

signal transduction pathway from the TCR to the nucleus. An increase of intracellular calcium, which may occur due to calcium influx or release from intracellular stores, indicates that this pathway has been activated. Intracellular calcium levels were therefore compared in iIEL and splenic lymphocytes in response to calcium ionophores which are known to cause a release of intracellular calcium in splenic and peripheral blood lymphocytes. Chicken splenic, duodenal and ileal lymphocytes, as well as mouse splenic and intestinal lymphocytes, were loaded with the calcium sensitive fluorochrome, fluo-3, in the presence of pluronic acid which enhances entry of the fluorescent dye into the cells. These cells were analyzed by flow cytometry for one minute to establish baseline fluorescence. They were quickly removed from the flow cytometer, a calcium ionophore was added and they were placed back on the instrument for further analysis. As calcium was released from intracellular stores or imported from the extracellular medium the fluorescence intensity was increased. As can be seen in Figures 9 and 10, splenic lymphocytes from both mouse and chicken responded normally to the calcium ionophores ionomycin and A23187 in that they demonstrated a dramatic increase in intracellular free calcium as indicated by a rise in fluorescent intensity over time. However, neither mouse nor chicken iIEL showed a significant increase in fluorescent intensity over time indicating that intracellular free calcium was not released in response to ionomycin or A23187.

This lack of response by iIEL is totally unexpected and no physiological basis is known for it. Attempts were made to rule out the possibility that this is due to an artifact of the iIEL isolation procedure because EDTA, a strong calcium chelator, is used during the preparation of the iIEL. EDTA was employed during the isolation of splenic cells

Figure 10

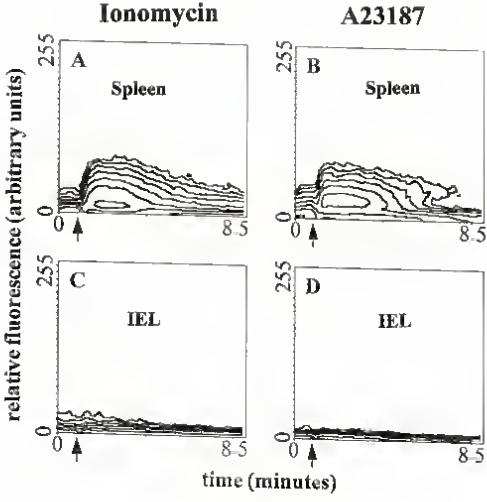
Release of intracellular calcium by mouse lymphocytes (mesenteric and splenic) after treatment with ionomycin and A23187. Units are relative arbitrary flourescence



50% Log
CH SPLEEN - IONO
CH DUO - IONO
CH ILEUM - IONO
CH SPLEEN - A23187
CH DUO - A23187
CH ILEUM - A23187
Time\Time (512 00 sec)-->

Figure 9

Release of intracellular calcium by chicken lymphocytes (duodenal ileal and splenic) after treatment with ionomycin and A23187 Units are relative arbitrary flourescence.


Ionomycin
A23187
A
Spleen
B
Spleen
C
IEL
D
IEL
255
0
0
8.5
relative fluorescence (arbitrary units)
time (minutes)

After treatment of the splenic cells with EDTA these cells remained responsive to stimulation with anti-CD3 and PMA plus ionomycin (Sanchez-Garcia 1996) EGTA, which does not cross the cell membrane was used to isolated iIEL When this modification of the isolation procedure was utilized the iIEL remained unresponsive to mitogenic stimulation (Sanchez-Garcia, 1996) Calcium mobilization was also measured with fura-2 loaded cells to determine if the variations in response were due to differential loading or other effects from use of this particular assay method iIEl were unresponsive under these assay conditions also (Sanchez Garcia, submitted)

The Refractory Phenotype of iIEL is not due to Overstimulation by Exogenous Antigen in the Gut

It has been postulated that the refractory phenotype of iIEL is due to the high amount of antigenic stimulation that they receive in the intestinal environment Studies to date using germ free mice have demonstrated that bacterial presence was not responsible for the lack of proliferation of these cells iIEL from germ free mice did not proliferate in response to mitogens (Regnault, 1996) However, these studies did not exclude the possibility that the stimulation these cells were receiving from the food that the mice ingested, albeit sterile, was causing the lack of proliferation The chicken animal model provides a unique opportunity to test this hypothesis by using iIEL and splenocytes from 20 day old embryos that had not received stimulation from exogenous food or environmental antigens

iIEL and splenocytes were harvested from 20 day old chicken embryos and subjected to proliferation and calcium release assays as described above Given the caveat the physiological basis for that lack of $Ca^{++}$ mobilization by adult iIEL is unknown, this

experiment was performed to determine whether this feature of iIEL is intrinsic to their phenotype, perhaps due to microenvironmental influences of the intestinal epithelium as opposed to exposure to food or environmental antigens As can be seen in Figure 11, splenic lymphocytes proliferated when placed in culture with Con A at 5 μg/ml with counts reaching 17,000 cpm These cells also proliferated when cultured with Con A at 10 μg/ml or plate-bound anti CD3 antibodies, with counts reaching 18,300 cpm and 23 700 cpm respectively These cells also proliferated in the control wells with media alone suggesting that they were in proliferative phase when isolated However, day 20 embryonic iIEL did not proliferate in response to any mitogen or in control wells suggesting that these cells are nonresponsive due to the environment of the intestine and not antigenic exposure

As can be seen in Figure 12, 20 day old chicken embryo splenic lymphocytes did release intracellular calcium in response to treatment with ionomycin and A23187 iIEL from these same embryos did not release intracellular calcium in response to these same calcium ionophores When EGTA was added to the medium to chelate extracellular calcium and allow only the measure of intracellular calcium release, the splenic lymphocytes responded with release of calcium These data suggest that the refractory nature of iIEL is not due overstimulation by exposure to exogenous antigen but rather a function of the environment of the intestinal epithelium

Altered Tyrosine Phosphorylation of iIEL Intracellular Signaling Molecules

To further characterize the mechanisms that may be involved in the lack of response to mitogenic stimulation by iIEL, the TCR signal transduction pathway that is

Figure 11

$^{3}$H thymidine incorporation by lymphocytes harvested from 20 day old chicken embryos and stimulated with Con A at 5 and 10 ug/ml and anti CD3 coated wells Splenocytes are represented by solid black bars and IELs are represented by white bars Error bars are ± S D

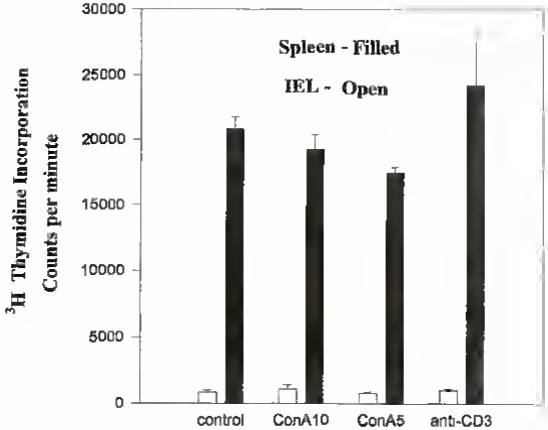
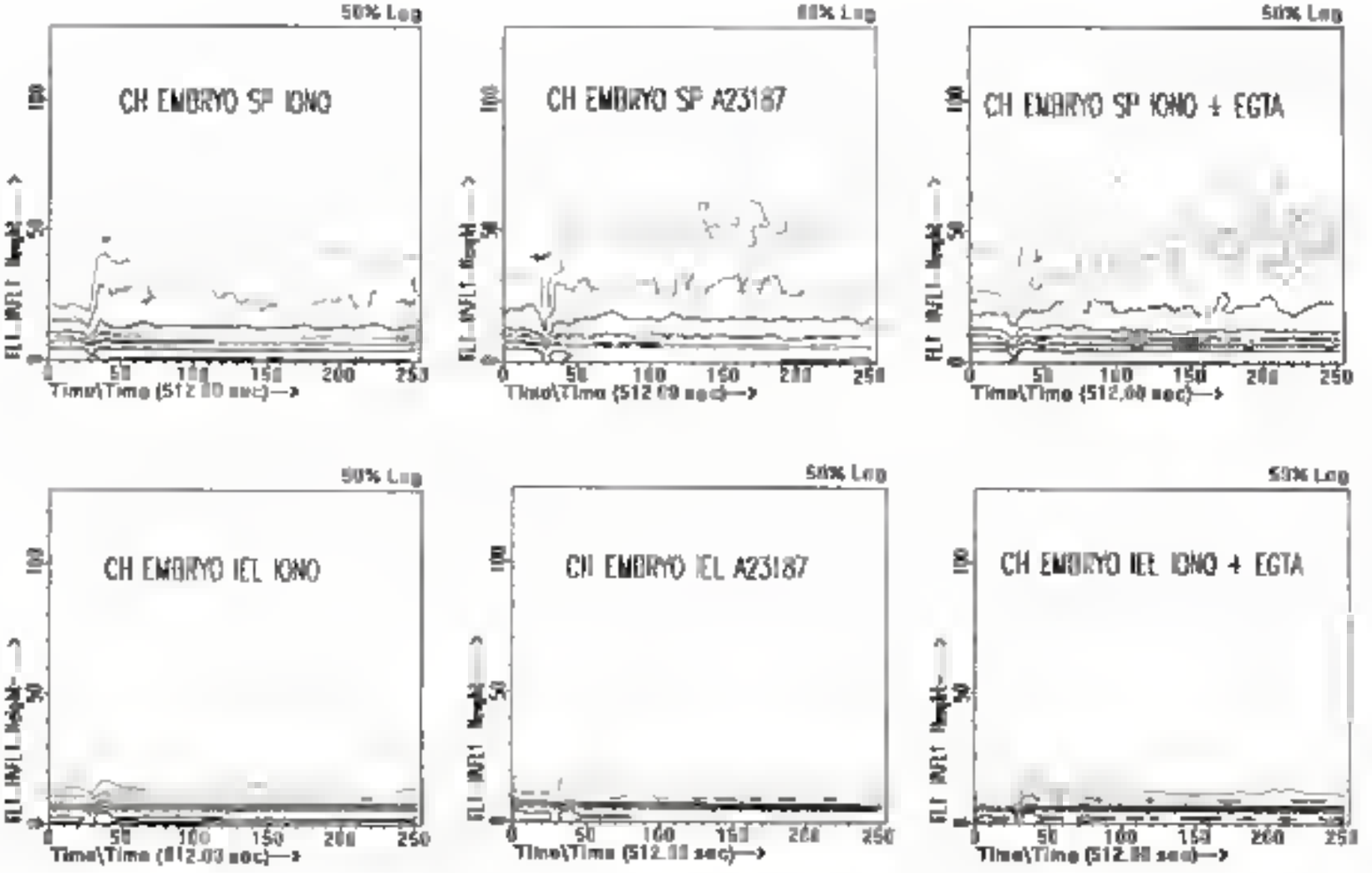

Spleen - Filled
IEL - Open
$^3$H Thymidine Incorporation
Counts per minute
30000
25000
20000
15000
10000
5000
0
control
ConA10
ConA5
anti-CD3

Figure 12

Intracellular calcium release by splenocytes and IELs harvested from 20 day old chicken embryos  Units are relative arbitrary flourescence


50% Leg
CH EMBRYO SP IONO
50% Leg
CH EMBRYO SP A23187
50% Leg
CH EMBRYO SP IONO + EGTA
50% Leg
CH EMBRYO IEL IONO
50% Leg
CH EMBRYO IEL A23187
50% Leg
CH EMBRYO IEL IONO + EGTA

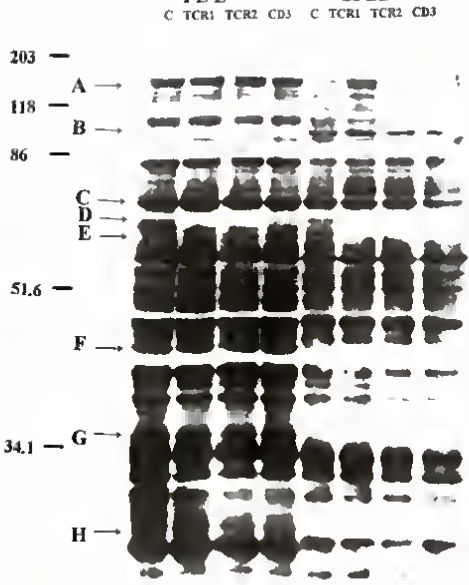
activated after TCR engagement was analyzed. To accomplish this, the mouse model was employed because there are many more reagents, such as monoclonal antibodies to signal transduction molecules, that are available for use in the mouse that do not exist for chickens. In splenocytes and peripheral blood lymphocytes several molecules are phosphorylated and others are dephosphorylated as the engagement signal is transduced from the TCR to the nucleus resulting in activation of T cells. This pathway can be mimicked in vitro by culturing isolated T cells with antibodies to TCR components and crosslinking these antibodies.

Mouse iIEL and splenocytes were harvested and incubated with antibodies to the γδ TCR (TCR1), αβ TCR (TCR2) and CD3ε for 1 hour at 4° C and then crosslinked with the appropriate anti-IgG and incubated at 37° C for 5 minutes. These cells were lysed in a buffer that inhibits kinase, phosphatase and protease activity, subjected to polyacrylamide gel electrophoresis (PAGE) and transferred to membranes. These membranes were then analyzed by western blot analysis for the presence of phosphorylated tyrosine residues using enhanced chemiluminescence per the manufacturer's protocol. As can be seen in Figure 13, there are a number of differences in the phosphorylation state of several molecules between iIEL and splenocytes prior to and following TCR crosslinking.

Position A on Figure 13 represents a molecule of molecular weight (MW) ~170-180 kD that is heavily constitutively phosphorylated in iIEL but not in splenocytes. It does, however, appear to be more heavily phosphorylated in spleen cells that have been stimulated with anti-TCR1. Position B represents a molecule of MW ~95-105 kD that is constitutively phosphorylated in spleen but is only phosphorylated in iIEL after stimulation.

Figure 13

Western blot analysis of tyrosine phosphorylated proteins. Lanes labelled IEL are from IEL. Lanes labelled spleen are from splenocytes. Lanes labelled c are control (unstimulated) Lanes labelled TCR1 were stimulated with anti γδ TCR antibody Lanes labelled TCR2 were stimulated with anti αβ TCR antibody and lanes labelled CD3 were stimulated with antiCD3 antibody


IEL
SPLEEN
C TCR1 TCR2 CD3 C TCR1 TCR2 CD3
203
118
86
51.6
34.1
A
B
C
D
E
F
G
H

with anti-TCR2 Position C represents a molecule of MW ~80-85 kD that is constitutively phosphorylated in iIEL but is not phosphorylated in spleen before or after stimulation Position D represents a molecule of MW ~70-75 phosphorylated in iIEL without stimulation or with stimulation with anti-CD3 only but is dephosphorylated after stimulation with either anti-TCR1 or anti-TCR2 This molecule appears to be constitutively phosphorylated in unstimulated spleen cells but becomes less phosphorylated after any stimulation treatment Position E represents a very interesting molecule that is constitutively phosphorylated in unstimulated spleen cells but becomes dephosphorylated after stimulation with any of the antibodies used in this experiment However for this molecule the exact opposite is true for iIEL It is dephosphorylated without stimulation but becomes phosphorylated when stimulated by any of these treatments Position F represents a molecule of MW ~ 43 kD that is phosphorylated constitutively before and after stimulation in iIEL and is not phosphorylated in spleen under any circumstances This molecule was later identified as ERK-2 and was not detected in spleen (Figure 14) Position G represents a molecule of MW ~ 35-37 kD that is phoshorylated under all circumstances in iIEL but not in spleen This may be due to it not being expressed in spleen as is the case with ERK-2 Position H represents a molecule of MW ~20-25 kD that also is not phosphorylated in spleen but is phosphorylated under all circumstances in iIEL This may also be due to differential expression as in ERK-2 The blot shown is a representative of three experiments

In summary, there are at least 8 distinct differences in the tyrosine phosphorylation patterns between iIEL and splenocytes In six cases (A,C,E,F,G,H) there is more

phosphorylation or expression in iIEL than in splenocytes, and in two cases (E,D) there is less phosphorylation in iIEL than in splenocytes. These results indicate that there is altered tyrosine phosphorylation between iIEL and splenocytes when stimulated via the TCR/CD3 complex.

Specific Signal Transduction Molecules are Differentially Expressed or Phosphorylated in iIEL as Compared to Splenocytes.

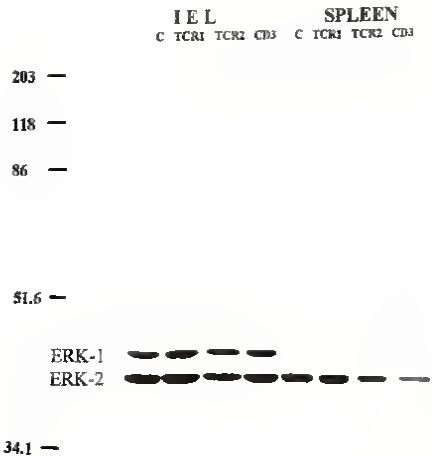
Because there are differences between iIEL and splenocytes in the above experiments, attempts were made to identify which molecules involved in signal transduction may be differentially expressed or phosphorylated. After stimulation and analysis as in the above experiments, the blots were stripped and reprobed with antibodies to specific signal transduction molecules. Alternatively, samples were run in duplicate and individual strips were probed with different antibodies. The panel of antibodies was selected based on the molecular weight of molecules that exhibited differential phosphorylation patterns and based on known molecules that are involved in T cell signal transduction. Six different antibodies were tested. The most intriguing and definitive data were obtained using anti-MAP 2 kinase, anti-Fyn and anti-ZAP 70.

MAP2 kinase

In this case the membrane was stripped and reprobed with a commercial antibody that recognizes both ERK-1 and ERK-2. As can be seen in Figure14, ERK 2 is expressed at similar levels in both iIEL and splenocytes. However, ERK 1 is expressed at much higher levels in iIEL than in spleen. ERK-1 is undetectable in spleen by this assay both before and after stimulation. Both of the ERKs were constitutively phosphorylated when

Figure 14

Western blot analysis of the MAP kinases ERK-1 and ERK-2 are recognized by this antibody that crossreacts with both Lanes labelled tIEL are from tIEL Lanes labelled spleen are from splenocytes Lanes labelled c are control (unstimulated) Lanes labelled TCR1 were stimulated with anti γδ TCR antibody Lanes labelled TCR2 were stimulated with anti αβ TCR antibody and lanes labelled CD3 were stimulated with anti CD3 antibody


IEL
SPLEEN
C TCR1 TCR2 CD3 C TCR1 TCR2 CD3
203
118
86
51.6
ERK-1
ERK-2
34.1

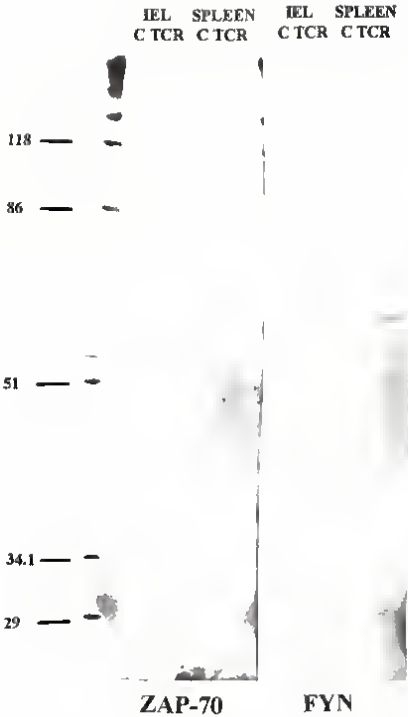
they were expressed by these cells as determined by probing with antiphosphotyrosine antibodies (Figure 13)

Fyn and ZAP-70

In this experiment the same four samples from iIEL and splenic cells that had been stimulated with antibodies to both TCR1 and TCR2 together (labelled TCR) or left unstimulated (labelled C) were run in adjoining lanes The membrane was cut into strips and exposed to the primary antibodies anti-ZAP-70 and anti-Fyn. The membranes were then incubated with peroxidase-conjugated secondary antibody and detected with ECL As can be seen in Figure 15, Fyn protein was present in equivalent amounts in both iIEL and splenocytes. However, ZAP 70 could only be detected in splenocytes

Figure 15

Western blot analysis for ZAP-70 and Fyn protein. Lanes labelled c were left unstimulated, Lanes labelled TCR were stimulated with antibodies to both αβ and γδ TCR.


IEL SPLEEN IEL SPLEEN
C TCR C TCR C TCR C TCR
118
86
51
34.1
29
ZAP-70
FYN

# CHAPTER 7
# DISCUSSION

The unique activation status of intestinal intraepithelial lymphocytes (iIEL) was investigated. These cells comprise a very large compartment of the immune system and are poorly understood. As the first set of cells capable of mounting an immune reponse to any ingested antigen and therefore providing the first line of defense against pathogens or toxins, a more complete understanding of why and how they may or may not become activated is of significant clinical and scientific relevance. The potential ability to enhance the response of the mucosal immune system could prove of great medical benefit to developing and developed nations, especially in children.

## Chickens May Serve as Good Animal Model to Study iIEL

Initial investigations involved using the chicken as an animal model. The chicken provides several advantages over mice: 1) They are much larger than mice and offer a much higher yield of iIEL per animal. The yield from a chicken is about $10^9$ iIEL/bird compared to ~5 x $10^6$ iIEL/mouse. 2) They are less expensive to purchase and maintain. Fertile chicken eggs cost about $10 per dozen and chickens can be maintained for about 5 cents per day in our own facility, compared to Balb/c mice which cost about $12 each and cost 18-22 cents per day to maintain. 3) Research on embryonic development is much more feasible. A 20-day-old chick embryo is about the same size as an adult mouse and it

was possible to harvest 3-6 x $10^5$ iIEL per embryo. There are no published reports at this time that iIEL have been harvested from mouse embryos.

Initial investigations were designed to determine the similarities and differences between iIEL phenotype and functional properties in chickens and mice. The mouse has been extensively studied and there exists much data to use as comparison. Flow cytometry was performed on chicken iIEL and splenocytes which served as controls. It was found that the majority of chicken iIEL were CD8$^+$ CD3$^+$ and about 50% γδ TCR and about 50% αβ TCR. These data are similar to mice (Mosley, 1994) (Table 1) and also similar to what has been found by other researchers studying the chicken model (Lillehoj, 1994). This not only confirmed that phenotypically this was a valid population for study but this also confirmed that the isolation procedures were adequate. To determine if these cells were functionally similar to iIEL from other species, $^3$H thymidine incorporation assays were performed as a measure of cellular proliferation in response to a variety of mitogens that are known to induce proliferation in other populations of lymphocytes such as PBL, splenic lymphocytes and lymph node lymphocytes. Results paralleled those found by other researchers (Sydora, 1994). iIEL from chickens did not proliferate in response to PMA, ionomycin, PMA plus ionomycin, or immobilized antibodies to the CD3 molecule. However, splenic lymphocytes responded as expected in that they proliferated in response to treatment with PMA plus ionomycin and to anti-CD3. PMA is an activator of protein kinase C, which in turn activates the Ras, Raf-1 pathway. Ionomycin is an electroneutral ionophore that exchanges one $Ca^{2+}$ for two $H^+$ ions and therefore, induces the discharge of $Ca^{2+}$ stores. Calcium ions then bind to the calcium

dependent protein phosphatase, calcineurin, which dephosphorylates the cytoplasmic subunit of Nuclear Factor of Activated T cells (NF AT) which then translocates to the nucleus and stimulates transcription These two pathways represent independent mechanisms for stimulating transcription These results imply that the lack of activation of iIEL may be due to differences in signal transduction steps downstream of the steps activated by PMA and ionomycin However there is evidence that other upstream pathways that are involved in signal transduction may also be different in iIEL

iIEL Constitutively Express Higher Granzyme B Levels than PBL.

There are several lines of evidence that indicate that iIEL are constitutively activated in vivo These include a larger and more granular morphology the finding that they can lyse target cells in a redirected cytotoxicity assay and the finding that they contain serine esterases that are released upon crosslinking of surface molecules involved in antigen recognition such as CD3 and TCR Granzyme B has been used as a marker for activated T cells in vitro (McElhaney 1996) The expression of this intracellular enzyme correlates with an activated and cytolytic phenotype To assess the activational state of iIEL using this enzyme as a marker for activation, granzyme B assays on iIEL and PBL were performed as described in Materials and Methods Fresh cell preparations and cells that had been cultured with Con A, which has been shown to induce activation and granzyme B expression in lymphocytes, were used The results in Figure 8 indicate that granzyme B is elevated in freshly isolated iIEL, relative to PBL, which is consistent with the finding that this cell population is constitutively activated After culture with Con A for 48 hours the granzyme B levels decrease in iIEL while they increase in PBL The

increase in granzyme B in PBLs was expected and the finding of a decrease in iIEL is not unexpected because most iIEL decrease their ability to mediate cell lysis after culture for significant periods of time (Sydora, 1994). The iIEL were 70-75% viable and the PBL were 90-95% viable after 48 hours as determined by trypan exclusion.

iIEL do not Mobilize Intracellular Calcium in Reponse to Calcium Ionophores

Because of the lack of a proliferative response to induction of calcium ions in iIEL by PMA plus ionomycin, the nature of calcium activity in iIEL and splenocytes was investigated. As previously reviewed, the release of intracellular calcium correlates with, and is responsible for, activation of cellular transcription. This release can be measured before and after stimulation with various treatments to ascertain the functional status of this activation pathway. iIEL and splenocytes were loaded with a dye (fluo-3) that flouresces when in contact with free intracellular calcium ions. Intracellular calcium release was measured by an increase in fluoresence over background. Chicken splenic lymphocytes released calcium in response to the addition of ionomycin and A23187. However, chicken iIEL did not release calcium in response to the addition of either one of these calcium ionophores. Mouse iIEL were tested for their response to the same calcium ionophores. Mouse splenocytes and iIEL behaved the same as chicken cells in that splenocytes released calcium in response to the two ionophores but iIEL did not. This lack of response to these ionophores indicates that these cells use a different activation pathway than other populations of lymphocytes. There may be several reasons for this such as a difference in the responsiveness of the calcium release channels on the endoplasmic reticulum that is responsible for release of calcium stores or differences in the

receptors for this release. There may also exist differences in the calcium release activated calcium (CRAC) channels in the plasma membrane that are responsible for the influx of calcium from the extracellular medium. This may be a critical observation. No other population of lymphocytes or any other cell type does not respond to these ionophores and the physiological basis for this is not known. iIEL have a distinctly different response to antigens and an inability to release intracellular stores of calcium may be one key to this lack of response to antigens. Because calcium ions bind to and activate the calcium dependent phosphatase, calcineurin, these results may indicate that the responsiveness of iIEL is altered at this more distal portion of the signal transduction pathway as well as at the proximal (TCR-PTK) end.

The Lack of in vitro Responsiveness is not due to Overstimulation by Exogenous Antigens

The lack of a proliferative response to antigenic stimulation in vitro exhibited by iIEL has been thought to be due to an antigenic overload caused by the immense amount of antigens that these cells are constantly exposed to in the intestinal environment. In the gut of the host these cells are constantly bombarded by antigens present in food as well as the microbial flora that is present in all intestinal environments. It has been hypothesized that this antigenic overstimulation is responsible for these cells inability to respond further to in vitro stimulation. This hypothesis has been tested only in mice where germ free mice were fed sterile food thereby eliminating any microbial antigenic exposure (Regnault, 1996). In these experiments the iIEL were still not responsive to mitogenic stimulation in vitro. However, these experiments did not address the possibility that the food that these mice were being fed was responsible for their inability to proliferate. These experiments

only addressed the question of bacterial presence. Investigations were undertakee to determine whether the preseece of exogenous antigen of any type, food or bacteria, was responsible for the nonresponsive phenotype of iIEL by using iIEL from 20-day old chicken embryos as a source of iIEL. These embryos are about the size of an adult mouse and yielded 3-6 x $10^6$ IEL per embryo. These cells were used in both proliferation assays and calcium release assays. The embryonic iIEL did not respond to Con A at either 5 or 10 ug/ml or to anti-CD3 coated wells. Splenocytes in all treatment groups proliferated well in this assay. Splenocytes also proliferated in the untreated group. This may be due the fact that they were just removed from the spleens of these embryos and were in the proliferative phase of ontogeny where they were increasing in number to become the resident populations of the spleen and periphery.

When treated with calcium ionophores, the iIEL from these embryos did not release intracellular calcium although there is no known basis for this observation. Ionomycin has been implicated as a compound responsible for opening the calcium release activated calcium (CRAC) channels in the cellular membrane as well as releasing calcium from intracellular stores (Fanger, 1995). The splenocytes responded normally in that a large increase in intracellular free calcium was observed in response to these treatments. Taken together, these data indicate that exogenous antigen exposure is not responsible for the nonresponsive phenotype of iIEL. This indicates that it may be the intestinal environment that causes this phenomenon either by secreted products or by direct cellular contact with the epithelial cells of the gut. These experiments do not rule out the

possibility that endogenous antigen, such as those that are processed by the embryo during development induce the nonresponsiveness in iIEL, but this seems unlikely

iIEL Exhibit Different Protein Tyrosine Phosphorylation Patterns from Splenocytes

iIEL recognize antigens via their TCR in a manner similar to other populations of T cells albeit in a more oligoclonal fashion, yet still fail to proliferate This raises the question of whether after recognition of antigen by the TCR, the intracellular mechanisms of passing this message to the nucleus and thereby stimulating proliferation is different between iIEL and other populations of lymphocytes This process of signal transduction has been well characterized in mouse splenic peripheral blood and lymph node T cells A large part of these pathways involves the phosphorylation of various molecules within the cytoplasm of cells as previously reviewed in Figures 2 and 3

The phosphorylation state of intracellular signalling molecules was analyzed before and after stimulation of iIEL and splenocytes with known TCR-stimulating regimens The mouse model was used for these studies because of the availability of antibodies to signal transduction molecules

There were at least eight distinct differences in the phosphorylation state or expression of phosphorylated proteins between iIEL and splenocytes Several of these differences appear to be differences in expression Bands C F G and H are present constitutively in iIEL but not at all in spleen. Thus, along with the finding that band F turns out to be expressed only in iIEL as discussed below, indicates that these molecules may only be expressed in iIEL However band A is constitutively phosphorylated in iIEL but only becomes phosphorylated in response to crosslinking of TCR1, a γδ TCR, whereas

band B is constitutively phosphorylated in spleen but only becomes phosphorylated in iIEL upon crosslinking of TCR2, an αβ TCR. This may indicate that these molecules are involved in some of the differences in responsiveness that are seen between these two populations (γδ vs αβ ) of cells in these different environments. Band D may also represent a molecule involved in differential signal transduction. It is constitutively phosphorylated in both populations of cells when unstimulated but becomes dephosphorylated in iIEL when these cells are stimulated with either TCR1 or TCR2. However, when iIEL are stimulated via CD3 this molecule remains phosphorylated. In spleen, this molecule becomes less phosphorylated when stimulated with any of the treatment regimens. Band E displays a very interesting phosphorylation pattern. This molecule is not phosphorylated in unstimulated iIEL but becomes phosphorylated when stimulated with any treatment used in this experiment. However, the exact opposite is true of spleen cells. This band is phosphorylated in unstimulated spleen cells but becomes dephosphorylated when stimulated with any treatment. This complete reversal of phosphorylation may provide some clues as to the mechanism of aberrant responsiveness in iIEL.

These differences indicate that iIEL use different signal transduction pathways than splenic lymphocytes. These differences may partially account for the lack of proliferation that is seen in these cells in vitro. It is of obvious benefit that iIEL do not proliferate in response to the huge variety of antigens that are encountered in the gut environment. Such a runaway immune response would certainly be detrimental to the host. However, these cells need to be able to recognize infected or damaged cells and indeed do expand

and multiply during infection. A different and unique signal transduction pathway would enable these cells to ignore signals from chronic antigen exposure (e.g. by food) that might cause other lymphocytes to proliferate while maintaining their ability to recognize antigens and respond in an appropriate fashion whether that was cytolysis, secretion, expansion or no response at all. This may be accomplished by the use of unique MHC molecules by the epithelial cells in the intestine for antigen presentation or by the use of different coreceptors by both iIEL and epithelial cells.

iIEL Express Different Signal Transduction Molecules from Splenocytes

After identifying different phosphorylation patterns in iIEL, specific signal transduction molecules that may be responsible for these differences were analyzed. To accomplish this, the blot was stripped and reprobed with antibodies to various specific signal transduction molecules. The probes used included antibodies to PKC, Lck, Fyn, CD3ζ, ZAP-70, and MAP-2 kinase. The first four antibodies PKC, Lck, Fyn and CD3ζ yielded no differences between iIEL and splenocytes. However, some interesting differences were noted with the antibody to the MAP-2 kinases. This antibody recognizes both ERK-1 and ERK-2 which are of MW 43kD and 41 kD respectively. ERK-2 was expressed at about the same levels in both iIEL and splenocytes. However, in this assay ERK-1 was detected only in iIEL. Both of these proteins were constitutively phosphorylated under these assay conditions. As discussed above, the MAP kinases are more distal in the signal transduction pathway and are capable of crossing the nuclear membrane to stimulate the cellular transcription machinery. In the case of peripheral T cells this results in the production of IL-2 and activation. It may be that the preferential

use of ERK 1 by iIEL leads to transcription of proteins involved in the activation of cytolytic effector mechanisms or other iIEL specific responses instead of the proliferative response seen in other populations of T cells. The MAP kinases have recently been implicated as signal transduction molecules that are at least partialy responsible for the lack of IL-2 production in cases of T cell anergy (Williams 1996). MAP kinase activity has been demonstrated to be down regulated in anergized CD4+ T cells leading to decreased Ras activity and decreased IL-2 transcription (Li, 1996). This block in Ras activation may result in an inability to engage the transcription factors Fos and Jun which drive activating protein 1 (AP-1)-modulated transcription of the IL-2 gene. This type of selective response would enable the iIEL to exert the proper effector functions such as cytolysis without initiating a runaway immune response. As has been previously reported, iIEL are constitutively cytotoxic in culture and have elevated MAP-2 kinase activity. These researchers also found comparable levels of expression of ERK-2 but did not examine the expression of ERK-1 (Sydora, 1993).

Fyn was present in nearly equal amounts in splenocytes and iIEL, however ZAP-70 was only detectable in splenocytes. This absence of detectable ZAP-70 in iIEL could contribute to the difference in the responsiveness of iIEL to in vitro stimulation. Because ZAP-70 is necessary to initiate a "normal" immune response the absence of this protein may be a factor in the hyporesponsiveness of iIEL in vitro. The absence of this proximal signal transduction molecule may serve to "uncouple" the TCR from the normal intracellular signalling machinery rendering iIEL unable to proliferate in response to TCR stimulation. ZAP-70 is essential for the normal reponse of T cells following TCR

stimulation Humans with a rare form of severe combined immunodeficiency have been shown to lack ZAP 70 ZAP-70 normally associates with the ζ chain of the CD3 complex following phosphorylation of the ζ chain by Lck after TCR stimulation ZAP-70 is then itself phosphorylated and dissociates from the ζ chain and phosphorylates other substrates which may include Vav Shc Grb-2 and others The lack of detectable levels of ZAP-70 in iIEL may signify that a different set of kinases, or another Syk/ZAP 70 family member with iIEL specific expression function under these conditions This could be part of the mechanism that allows iIEL to recognize antigens via the TCR and exert a cytotoxic effect without proliferating.

So far two signal transduction molecules have been identified that are differentially regulated in iIEL as compared to splenic lymphocytes One of these ZAP 70 functions at the proximal end of the signal cascade by interacting with the TCR-CD3 complex at the T-cell membrane The other ERK-1, functions at the most distal end of the signal cascade and translocates to the nucleus to stimulate cellular transcription Because of these two fundamental differences at widely disparate ends of the signal cascade, it would seem that there probably exists other differences as well

# CHAPTER 8
# CONCLUSIONS

## IEL Function and Phenotype is Evolutionarily Conserved Between Chickens and Mammals

This was demonstrated by the similarities in the flow cytometry profiles the lack of response to mitogens and the lack of mobilization of intracellular calcium in response to calcium ionophores by both mouse and chicken IEL

## The Lack of Proliferation in Response to Stimulation by Specific and Nonspecific Mitogens May not be Due to Overstimulation by Exogenous Antigen.

This was demonstrated by the lack of proliferation and lack of calcium mobilization by IIEL harvested from 20-day-old chicken embryos that had never been exposed to exogenous antigen

## IIEL May Have Impaired Calcium Signalling Pathways.

This was demonstrated by the lack of response by IIEL to calcium ionophores which normally induce the release of intracellular calcium

## IIEL Have Altered Phosphorylation Patterns of Signal Transduction Molecules

This was demonstrated by western blot analysis of stimulated and unstimulated IIEL and splenocytes using antiphosphotyrosine antibodies that revealed distinct differences in the phosphorylation states of several intracellular proteins

<u>iIEL Differentially Express Certain Signal Transduction Molecules.</u>

This was demonstrated by western blot analysis using specific antibodies to signal transduction molecules and finding ERK 1 expressed in iIEL and not in splenocytes and finding ZAP-70 expressed in splenocytes and not in iIEL

# CHAPTER 9
# FUTURE DIRECTIONS

These studies are some of the first to characterize the molecular nature of the differences between iIEL and other populations of lymphocytes. While these studies have implicated certain mechanism that may be responsible for the unique activational status of iIEL, there are many questions that remain unanswered. Using the mouse system to study iIEL has some distinct advantages in this pursuit. There are many avenues to explore some of which are briefly discussed below.

1) Some initial investigations would involve the use of mouse epithelial cell lines as targets for iIEL in cytotoxicity experiments. It has been established that iIEL are capable of lysing Fc receptor positive cell lines in a redirected cytotoxicity experiment but this is quite different from what happens in vivo. Because there exist several cell lines of mouse epithelial origin, it would be informative to use these as targets for freshly isolated iIEL. These cells should be subjected to a number of different treatments to determine if any of these would render them subject to lysis by iIEL. These treatments should include infecting them with several intracellular pathogens of both viral and bacterial origin, subjecting them to heat stress to induce production of heat shock proteins (a putative target of iIEL), and possibly transfecting them with plasmids encoding other possible targets for iIEL. Although there are not any cell lines already established that are of intestinal origin, a great deal of information could be gleaned from using other epithelial

cell lines If certain treatments or infections rendered the line susceptible to lysis then these treatments could be explored further Also it would not be very difficult to establish intestinal epithelial cell lines This could be done in vitro using the myriad of transforming viruses and plasmids available for use in mice or in vivo by feeding mice highly carcinogenic substances and harvesting tumors that develop in the mouse intestine and culturing these to establish cell lines Alternatively, mouse epithelial cells could be cultured directly from the intestines of germ-free mice The problem that existed when working with chickens was the constant huge bacterial load associated with the intestinal epithelium, but this problem would be easy to avoid using germ free mice in the sterile housing conditions at the animal research facility These epithelial cells should not be that difficult to culture under these circumstances and has already been done using germ-free rats (Quaroni, 1979) If lysis was demonstrated during these experiments it would be useful to block these interactions using antibodies to cell surface molecules mediating these functions, such as MHC TCR, various coreceptors, Fas or FasL, and others This would prove conclusively that these molecules are involved in these lytic interactions

2) The use of iIEL cell lines to investigate their cytolytic potential and signal transduction machinery should not be extremely difficult because iIEL cell lines have already been established from mice. However the focus of study on these lines so far has been on their antigen recognition capabilities and not on the molecular nature of their signal transduction pathways Applying the same type of investigations already used in this study would allow scientists to investigate these cell lines more thoroughly Cell lines

tend to yield cleaner data even if conclusions may only apply to the class of cells being studied

These cell lines could be used in a variety of functional assays including cytolytic and proliferative assays (although proliferation studies are typically more difficult to interpret when using a continuously growing cell line, elevation of proliferation rate above background or baseline proliferation can be assessed)

3) Investigations of the nuclear machinery involved in cellular activation could be carried out It could be very useful to investigate iIEL activation from the nucleus out rather than from the cell surface in. Study of the nuclear transcription factors normally associated with IL-2 production such as NF-ATc and NF ATn and using chimeric molecules or transcriptionally activated NF-AT that would bind to the IL-2 promoter and recruit known transcriptional activating factors could induce proliferation of iIEL. If these iIEL could be induced to proliferate using this scheme then analysis of the factors and events upstream of nuclear activation could be performed

4) Co-culture of iIEL with conditioned media from spleen cell and tissue culture would help define the nature of the environment eliciting the altered responsiveness of IIEL Splenocytes should be isolated and cultured with and without Con A and other stimulating agents This should be done with and without the presence of the spleen itself in the culture All of the above treatments should be done with normal media containing fetal bovine serum (FBS) and also with serum-free media. These media could then be used to culture freshly isolated iIEL in proliferation assays These conditions would mimic

the spleen environment and possibly allow the tIEL to proliferate. If the tIEL proliferated the factors associated with this proliferation could be assessed.

5) Co-culture of splenic or peripheral T cells with intestinal tissue from germ-free mice may induce the lack of responsiveness. One of the major problems associated with trying to reproduce the intestinal environment is the high bacterial load that accompanies this tissue. By using germ-free mice it would be possible to place intestinal sections in culture and inject these pieces with splenic or peripheral blood lymphocytes. The need to ligate one or both ends of the intestine to keep the cells inside may arise. After 2-3 days in culture these cells could be removed and tested for the ability to proliferate to determine if this had been affected by the intestinal environment. This same type of experiment could also be done in vivo. By ligating the distal portion of a mouses intestine and injecting splenocytes in the proximal portion, splenocytes could be exposed to the intestinal environment. These cells would need to be labelled so as to be able to identify them and separate them from tIEL after isolation. Of course, the mice would have to be fed intravenously during this experiment. This experiment would tell us if the environment of the intestine is capable of rendering normal lymphocytes nonreactive.

6) To more closely examine cytolytic effector mechanism involved in tIEL-mediated cytotoxicity, analysis of Fas involvement should be undertaken using flow cytometry to determine if tIEL are Fas ligand (FasL) positive and if epithelial cells are Fas positive. Next, if cytolysis been induced, this lysis should be blocked by using anti Fas and anti FasL antibodies. These studies would increase our understanding of the way that tIEL mediate cytotoxicity of epithelial cells in the intestine.

7) Immunoprecipitation studies of signal transduction molecules using rats instead of mice would allow isolation of sufficient numbers of tIEL for these experiments When this was attempted in mouse tIEL the low yield of tIEL prevented definative immunoprecipitation studies (data not shown) Many of the signal transduction molecules form noncovalent associations during the process of passing the message from the cell surface to the nucleus The use of different strength detergents would allow a greater understanding of the associations and affinities that these molecules have for each other These can be immunoprecipitated using antibodies that are bound to sepharose beads or other matrices By using antibodies to one molecule and finding other molecules associated with it by western blot analysis it can be determined if these molecules associate with each other either before, during or after stimulation of cell surface receptors Many of these associations are known for peripheral blood and splenic lymphocytes. Studies of intramolecular associations in tIEL to determine if these same or different molecules associate with one another under different stimulation regimens would enhance our understanding of the nature of these cells Once these signal transduction PTKs have been immunoprecipitated, the kinase activity could be examined by incubating these proteins with different substrates and $^{32}$P-ATP and visualizing products with autoradiography

8) Time courses of both associations and phosphorylation states should be done These should be done under the conditions described above to determine the kinetics of intramolecular association These studies could be performed at lowered temperatures to slow down the reactions and allow a more precise assessment of the associations involved

## LIST OF REFERENCES


Abraham N, Veillette A (1990) Activation of p56lck through mutation of a regulatory carboxy terminal tyrosine residue requires intact sites of autophosphorylation and myristylation. Mol-Cell-Biol 10: 5197-206

Alberola Ila J, Hogquist KA, Swan KA, Bevan MJ, Perlmutter RM (1996) Positive and negative selection invoke distinct signaling pathways. J-Exp-Med 184: 9-18

Anderson P, Blue ML, Schlossman SF (1988) Comodulation of CD3 and CD4. Evidence for a specific association between CD4 and approximately 5% of the CD3-T cell receptor complexes on helper T lymphocytes. J-Immunol 140: 1732-7

Appleby MW, Gross JA, Cooke MP, Levin SD, Qian X, Perlmutter RM (1992) Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. Cell 70: 751-63

August A, Dupont B (1995) Activation of extracellular signal-regulated protein kinase (ERK/MAP kinase) following CD28 cross-linking: activation in cells lacking p56lck. Tissue Antigens 46: 155-62

August A, Dupont B (1956) Association between mitogen-activated protein kinase and the zeta chain of the T cell receptor (TCR) with the SH2-3 domain of p56lck: Differential regulation by TCR crosslinking. J-Biol-Chem 271: 10054-9

Baniyash M, Hsu VW, Seldin MF, Klausner RD (1989) The isolation and characterization of the murine T cell antigen receptor zeta chain gene. J-Biol-Chem 264: 13252-7

Barber EK, Dasgupta JD, Schlossman SF, Trevillyan JM, Rudd CE (1989) The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. Proc-Natl-Acad-Sci-U-S-A 86: 3277-81

Beagley KW, Fujihashi K, Black CA, Lagoo AS, Yamamoto M, McGhee JR, Kiyono H (1993) The *Mycobacterium tuberculosis* 71-kDa heat-shock protein induces proliferation and cytokine secretion by murine gut intraepithelial lymphocytes. Eur-J-Immunol 23: 2049-52

Berridge MJ, Irvine RF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312: 315-21

Burgess KE, Odysseos AD, Zalvan C, Druker BJ, Anderson P, Schlossman SF, Rudd CE (1991) Biochemical identification of a direct physical interaction between the CD4 p56lck and Ti(TcR)/CD3 complexes. Eur-J-Immunol 21: 1683-8

Caron L Abraham N Pawson T Veillette A (1992) Structural requirements for enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase p56lck Mol-Cell-Biol 12 2720 9

Chijiwa B Schneck JP Brummel ME Marsh DG Rafnar T (1996) Csk is constitutively associated with a 60-kDa tyrosine-phosphorylated protein in human T cells. J-Biol-Chem 271 5698 703

Chan AC Desai DM, Weiss A (1994) The role of protein tyrosine kinases and protein tyrosine phosphotases in T cell antigen receptor signal transduction Annu Rev Immunol 12 555-92

Chan AC, Irving BA, Fraser JD, Weiss A (1991) The zeta chain is associated with a tyrosine kinase and upon T cell antigen receptor stimulation associates with ZAP-70 a 70-kDa tyrosine phosphoprotein Proc-Natl-Acad-Sci U S A 88 9166-70

Chan AC Iwashima M, Turck CW Weiss A (1992) ZAP-70 a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain Cell 71 649-62

Cooke MP Abraham KM Forbush KA, Perlmutter RM (1991) Regulation of T cell receptor signaling by a src family protein tyrosine kinase (p59fyn) Cell 65 281-91

Croitoru K, Bienenstock J, Ernst PB (1994) Phenotypic and functional assessment of intraepithelial lymphocytes bearing a forbidden alpha beta TCR Int-Immunol 6 1467-73

Dembic Z Haas W Weiss S McCubrey J Kiefer H, von Boehmer H, Steinmetz M (1986) Transfer of specificity by murine alpha and beta T-cell receptor genes Nature 320 232-8

Doyle C Strominger JL (1987) Interaction between CD4 and class II MHC molecules mediates cell adhesion Nature 330 256 9

Elder ME, Hope TJ Parslow TG Umetsu DT Wara DW Cowan MJ (1995) Severe combined immunodeficiency with absence of peripheral blood CD8+ T cells due to ZAP-70 deficiency Cell Immunol 165 110-7

Fanger CM Hoth M Crabtree GR, Lewis RS (1995) Characterization of T cell mutants with defects in capacitive calcium entry genetic evidence for the physiological roles of CRAC channels J Cell Bio 131 655 667

Fields PE, Gajewski TF Fitch FW (1996) Blocked ras activation in anergic CD4+ T cells Science 271 1276-8

Frank SJ Samelson LE, Klausner RD (1990) The structure and signalling functions of the invariant T cell receptor components Semin Immunol 2 89 97

Fujiura Y Kawaguch M Kondo Y, Ob[illegible] S Yamamoto H Nanno M Ishikawa H (1994) Development of CD8 alpha alpha intestinal intraepithelial T cells in beta 2 microglobulin- and/or TAP1-deficient mice J Immunol 156 2710 5

Fung Leung WP Kundig TM, Ngo K, Panakos J De Sousa Hitzler J, Wang E, Ohashi PS, Mak TW, Lau CY (1994) Reduced thymic maturation but normal effector function of CD8+ T cells in CD8 beta gene-targeted mice J Exp Med 180 959-67

Furukawa T Itoh M Krueger NX, Streuli M, Saito H (1994) Specific interaction of the CD45 protein tyrosine phosphatase with tyrosine-phosphorylated CD3 zeta chain Proc Natl Acad Sci U S A 91 10928-32

Gauen LK, Zhu Y, Letourneur F Hu Q Bolen JB Matis LA, Klausner RD Shaw AS (1994) Interactions of p59fyn and ZAP-70 with T cell receptor activation motifs defining the nature of a signaling motif Mol Cell Biol 14 3729-41

Gelfanov V Lai YG Gelfanova V, Dong JY Su JP Liao NS (1995) Differential requirement of CD28 costimulation for activation of murine CD8+ intestinal intraepithelial lymphocyte subsets and lymph node cells J Immunol 155 76-82

Gibson S August A, Kawakami Y, Kawakami T Dupont B Mills GB (1996) The EMT/ITK/TSK (EMT) tyrosine kinase is activated during TCR signaling LCK is required for optimal activation of EMT J Immunol 156 2716-22

Glaichenhaus N Shastri N Littman DR, Turner JM (1991) Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells Cell 64 511 20

Godson DL Campos M, Babink LA (1992) The role of bovine intraepithelial leukocyte-mediated cytotoxicity in enteric antiviral defense Viral Immunol 5 1-13

Guy Grand D Rocha B Mintz F Malassis Seris M Selz F Malissen B Vassalli P (1994) Different use of T cell receptor transducing modules in two populations of gut intraepithelial lymphocytes are related to distinct pathways of T cell differentiation J Exp Med 180 673-9

Hermans MH, Malissen B (1993) The cytoplasmic tail of the T cell receptor zeta chain is dispensable for antigen mediated T cell activation Eur J Immunol 23 2257 62

Hettmann T Cohen A (1995) Identification of an ionomycin/cyclosporin A-responsive element within the human T cell receptor gamma enhancer Eur J Immunol 25 3356-64

Hui ED Siegel JN Minami Y, Luong ET Klausner RD Samelson LE (1989) T cell activation induces rapid tyrosine phosphorylation of a limited number of cellular substrates J Biol Chem 264 10836-42

Huleatt JW Lefrancois L (1995) Antigen driven induction of CD11e on intestinal intraepithelial lymphocytes and CD8+ T cells in vivo J-Immunol 154 5684-93

Imaoka A, Matsumoto S Setoyama H, Okada Y Umesaki Y (1996) Proliferative recruitment of intestinal intraepithelial lymphocytes after microbial colonization of germ free mice. Eur-J-Immunol 26 945 8

Irving BA, Chan AC Weiss A (1993) Functional characterization of a signal transducing motif present in the T cell antigen receptor zeta chain J Exp Med 177 1093 103

Isakov N Wange RL, Samelson LE (1994) The role of tyrosine kinases and phosphotyrosine-containing recognition motifs in regulation of the T cell antigen receptor-mediated signal transduction pathway J Leukoc-Biol 55 265-71

Isakov N Wange RL, Watts JD Aebersold R, Samelson LE (1996) Purification and characterization of human ZAP 70 protein-tyrosine kinase from a baculovirus expression system J-Biol-Chem 271 15753-61

Ishikawa H Li Y Abelkovich A, Yamamoto S Kaufmann SH Tonegawa S (1993) Cytotoxic and interferon gamma-producing activities of gamma delta T cells in the mouse intestinal epithelium are strain dependent Proc-Natl-Acad-Sci U-S-A 90 8204-8

Iwashima M, Irving BA, van Oers NS Chan AC Weiss A (1994) Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases Science 263 1136-9

Jayaraman T, Ondrias K, Ondriasova E, Marks AR (1996) Regulation of the inositol 1,4,5-trisphosphate receptor by tyrosine phosphorylation Science 272 1492-4

Kawakami T Pennington CY Robbins KC (1986) Isolation and oncogenic potential of a novel human src-like gene Mol Cell-Biol 6 4195-201

Kawamoto Y Sasaki K, Kato Y Kojima K, Tsuji T Miyama A (1996) Rapid killing of murine lymph node T blasts by intestinal intraepithelial lymphocytes in vitro Eur J-Immunol 26 653-8

Kelleher D Murphy A, Lynch S O'Farrelly C (1994) Adhesion molecules utilized in binding of intraepithelial lymphocytes to human enterocytes Eur J Immunol 24 1013-6

Kim HK, Kim JW Zilberstein A, Margolis B, Kim JG Schlessinger J Rhee SG (1991) PDGF stimulation of inositol phospholipid hydrolysis requires PLC-gamma 1 phosphorylation on tyrosine residues 783 and 1254 Cell 65 435-41

Kim KM Alber G Weiser F, Reth M (1993) Differential signaling through the Ig-alpha and Ig beta components of the B cell antigen receptor Eur J Immunol 23 911 6

Kissinger CR, Parge HE, Knighton DR, Lewis CT Pelletier LA, Tempczyk A, Kalish VJ Tucker KD Showalter RE, Moomaw EW, et al (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex Nature 378 641-4

Kolanus W Romeo C Seed B (1993) T cell activation by clustered tyrosine kinases Cell 74 171-83

Labadia ME Ingraham RH Schembri King J, Morelock MM, Jakes S (1996) Binding affinities of the SH2 domains of ZAP-70, p56lck and Shc to the zeta chain ITAMs of the T-cell receptor determined by surface plasmon resonance J-Leukoc-Biol 59 740-6

Lefrancois L, Barrett TA, Havran WL, Poddington L (1994) Developmental expression of the alpha IEL beta 7 integrin on T cell receptor gamma delta and T cell receptor alpha beta T cells Eur J-Immunol 24 635-40

Lefrancois L, Olson S (1994) A novel pathway of thymus-directed T lymphocyte maturation J-Immunol 153 987-95

Letourneur F, Gabert J Cosson P, Blanc D Davoust J, Malissen B (1990) A signaling role for the cytoplasmic segment of the CD8 alpha chain detected under limiting stimulatory conditions Proc-Natl-Acad-Sci-U-S-A 87 2339-43

Letourneur F, Klausner RD (1992) Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 epsilon Science 255 79 82

Li W Whaley CD Mondino A, Mueller DL (1996) Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4 T cells. Science 271 1272-1276

Lillehaug HS (1993) Avian gut-associated immune system implication in coccidial vaccine development Poult Sci 72 1306-11

Lillehoj HS (1994) Analysis of *Eimeria acervulina*-induced changes in the intestinal T lymphocyte subpopulations in two chicken strains showing different levels of susceptibility to coccidiosis Res Vet Sci 56 1 7

Lillehoj HS, Bacon LD (1991) Increase of intestinal intraepithelial lymphocytes expressing CD8 antigen following challenge infection with *Eimeria acervulina* Avian Dis 35 294-301

Lin T Matsuzaki G Kenai H, Kishihara K, Nabeshima S Fung Leung WP, Mak TW, Nomoto K (1994a) Characteristics of fetal thymus-derived T cell receptor gamma delta intestinal intraepithelial lymphocytes Eur J Immunol 24 1792-8

Lin T Matsuzaki G Kenai H, Nakamura T Nomoto K (1993) Thymus influences the development of extrathymically derived intestinal intraepithelial lymphocytes Eur J Immunol 23 1968-74

Lin T Matsuzaki G Yoshida H Kobayeshi N Kenai H, Omoto K, Nomoto K (1994b) CD3 CD8+ intestinal intraepithelial lymphocytes (IEL) and the extrathymic development of IEL Eur J-Immunol 24 1080-7

Liu B Hackshaw KV, Whisler RL (1996) Calcium signals and protein tyrosine kinases are required for the induction of c-jun in Jurkat cells stimulated by the T cell receptor complex and oxidative signals J Interferon Cytokine Res 16 77-90

McElhaney JE, Pinkoski MJ Upshaw CM Bleakley RC (1996) The cell mediated cytotoxic response to influenza vaccination using as essey for granzyme B activity J Immunol Methods 190 11 20

Mittler RS Goldman SJ Spitalny GL Burakoff SJ (1989) T-cell receptor-CD4 physical association in a murine T cell hybridoma induction by antigen receptor ligation Proc-Natl-Acad Sci U-S-A 86 8531-5

Molina TJ Kishihara K, Siderovski DP, van Ewijk W Narendran A, Timms E Wakeham A, Paige CJ Hartmann KU Veillette A, et al (1992) Profound block in thymocyte development in mice lacking p56lck Nature 357 161-4

Mosley RL Klein JR (1992) A rapid method for isolating murine intestine intraepithelial lymphocytes with high yield and purity J Immunol-Methods 156 19 26

Mosley RL Wang J Hamad M, Klein JR (1994a) Functional heterogeneity of murine intestinal intraepithelial lymphocytes studies using TCR alpha beta+ IEL lines and fresh IEL isolates reveal multiple cytotoxic subsets differentiated by CD5, CD8 alpha/alpha, and CD8 alpha/beta expression Dev-Comp Immunol 18 155-64

Mosley RL, Whetsell M, Stickney D Whetsell L Schaefer FV Miller KS Klein JR (1994b) Phenotype and TCR gamma delta variable gene repertoire of intestinal intraepithelial lymphocytes in wild mice (*Mus musculus domesticus*) abundance of V gamma 1 transcripts and extensive delta gene diversity Int-Immunol 6 231-8

Nanno M, Matsumoto S Koike R, Miyasaka M, Kawaguchi M, Mesude T Miyewaki S, Cai Z Shimamura T Fujiura Y, et al (1994) Development of intestinal intraepithelial T lymphocytes is independent of Peyer s patches and lymph nodes in aly mutant mice J Immunol 153 2014-20

Neuhaus O Emoto M, Blum C, Yamamoto S, Kaufmann SH (1995) Control of thymus-independent intestinal intraepithelial lymphocytes by beta 2-microglobulin Eur-J-Immunol 25 2332-9

Ni J Hollander D (1995) Expression of beta 7 integrins and other cell adhesion molecules on mouse lymphocytes and their modulation by a new cytokine IL-2 receptor-inducing factor Cell Immunol 164 150-5

Ni J Hollander D, Sydora B Panwala C (1995) Adhesion molecule expression and adhesion properties of murine intestinal intraepithelial lymphocyte hybridomas Cell Immunol 164 156-60

Normant AM Salter RD Parham P, Engelhard VH, Littman DR (1988) Cell cell adhesion mediated by CD8 and MHC class I molecules Nature 336 79 81

Ohteki T. MacDonald HR (1993) Expression of the CD28 costimulatory molecule on subsets of murine intestinal intraepithelial lymphocytes correlates with lineage and responsiveness Eur J-Immunol 23 1251-5

Park DJ Rho HW, Rhee SG (1991) CD3 stimulation causes phosphorylation of phospholipase C-gamma 1 on serine and tyrosine residues in a human T-cell line Proc-Natl Acad Sci-U-S A 88 5453-6

Penney L Kilshaw PJ MacDonald TT (1995) Regional variation in the proliferative rate and lifespan of alpha beta TCR and gamma delta TCR intraepithelial lymphocytes in the murine small intestine Immunology 86 212-8

Pereira P Gerber D, Huang SY, Tonegawa S (1995) Ontogenic development and tissue distribution of V gamma 1-expressing gamma/delta T lymphocytes in normal mice J-Exp Med 182 1921-30

Perlmutter RM Marth JD Lewis DB Peet R, Ziegler SF Wilson CB (1988) Structure and expression of lck transcripts in human lymphoid cells J-Cell-Biochem 38 117 26

Plas DR, Johnson R, Pingel JT Matthews RJ Dalton M, Roy G Chan AC Thomas ML (1996) Direct regulation of ZAP-70 by SHP 1 in T cell antigen receptor signaling Science 272 1173-6

Pluschke G Taube H, Krawinkel U Pfeffer K, Wagner H, Classen M Deusch K (1994) Oligoclonality and skewed T cell receptor V beta gene segment expression in in vivo activated human intestinal intraepithelial T lymphocytes Immunobiology 192 77-93

Puddington L, Olson S Lefrancois L (1994) Interactions between stem cell factor and c Kit are required for intestinal immune system homeostasis Immunity 1 733-9

Qian D Griswold Prenner I, Rosner MR, Fitch FW (1993) Multiple components of the T cell antigen receptor complex become tyrosine phosphorylated upon activation J-Biol-Chem 268 4488-93

Quarom A, Wands J Trelstra RL Isselbacher KJ (1979) Epithelioid cell cultures from rat small intestine Characterization by morphologic and immunologic criteria J-Cell Biol 80 248-65

Raab M, Rudd CE (1996) Hematopoietic cell phosphatase (HCP) regulates p56LCK phosphorylation and ZAP-70 binding to T cell receptor zeta chain Biochem-Biophys-Res Commun 222 50 7

Regnault A, Cumano A, Vassalli P Guy Grand D Kourilsky P (1994) Oligoclonal repertoire of the CD8 alpha alpha and the CD8 alpha beta TCR alpha/Beta murine intestinal intraepithelial T lymphocytes evidence for the random emergence of T cells J-Exp-Med 180 1345-58

Roberts AI O Connell SM Biancone L, Brolin RE Ebert EC (1993a) Spontaneous cytotoxicity of intestinal intraepithelial lymphocytes clues to the mechanism Clin-Exp-Immunol 94 527 32

Roberts AI O Connell SM, Ebert EC (1993b) Intestinal intraepithelial lymphocytes bind to colon cancer cells by HML 1 and CD11a Cancer-Res 53 1608-11

Roberts K, Kilshaw PJ (1993) The mucosal T cell integrin alpha M290 beta 7 recognizes a ligand on mucosal epithelial cell lines Eur-J-Immunol 23 1630-5

Roebo B Vassalli P, Guy Grand D (1994) Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice J Exp-Med 180 681-6

Rudd CE, Trevillyan JM, Dasgupta JD, Wong LL, Schlossman SF (1988) The CD4 receptor is complexed in detergent lysates to a protein tyrosine kinase (pp58) from human T lymphocytes Proc Natl-Acad-Sci U-S-A 85 5190-4

Russell GJ Parker CM, Cepek KL Brenner MB Bhan AK (1994) Evidence for a structural difference in the CD7 polypeptide on human thymocytes and intraepithelial lymphocytes defined by a new monoclonal antibody 3D9 Cell-Immunol 154 153-65

Samelson LE, Patel MD Weissman AM, Harford JB Klausner RD (1986) Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor Cell 46 1083-90

Samelson LE, Phillips AF Luong ET Klausner RD (1990) Association of the fyn protein tyrosine kinase with the T-cell antigen receptor Proc Natl Acad-Sci U-S-A 87 4358-62

Sanchez Garcia FJ, Aller WW McCormack WT (1996) Impaired calcium mobilization and differential tyrosine phosphorylation in intestinal intraepithelial lymphocytes submitted to Int Immunol

Sasahara T Tamauchi H, Ikewaki N Kubota K (1994) Unique properties of a cytotoxic CD4+CD8+ intraepithelial T-cell line established from the mouse intestinal epithelium Microbiol-Immunol 38 191 9

Secrist JP Karnitz L, Abraham RT (1991) T cell antigen receptor ligation induces tyrosine phosphorylation of phospholipase C-gamma 1 J-Biol-Chem 266 12135-9

Semba K, Nishizawa M, Miyajima N Yoshida MC, Sukegawa J Yamanashi Y Sasaki M Yamamoto T Toyoshima K (1986) yes-related protooncogene, syn, belongs to the protein-tyrosine kinase family Proc-Natl-Acad-Sci-U S-A 83 5459-63

Shaw AS Amrein KE, Hammond C, Stern DF Sefton BM, Rose JK (1989) The lck tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain Cell 59 627-36

Shaw AS Chalupny J Whitney JA, Hammond C, Amrein KE, Kavathas P Sefton BM, Rose JK (1990) Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino- terminal domain of the p56lck tyrosine protein kinase Mol-Cell Biol 10 1853-62

Stebbecht MS Hsia E, Spyopalski C, Nagler Anderson C (1993) Stimulation of murine intestinal intraepithelial lymphocytes by the bacterial superantigen staphylococcal enterotoxin B Int-Immunol 5 717-24

Sperber K, Silverstein L, Brusco C, Yoon C, Mullin GE, Mayer L (1995) Cytokine secretion induced by superantigens in peripheral blood mononuclear cells lamina propria lymphocytes and intraepithelial lymphocytes Clin Diagn Lab Immunol 2 473 7

Steffan NM, Bren GD Frantz B Tocci MJ D Neill EA, Paya CV (1995) Regulation of IkB alpha phosphorylation by PKC- and Ca(2+)-dependent signal transduction pathways J Immunol 155 4685-91

Stern PL, Lee HM Roth S, Soriano P (1992) pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells Cell 70 741-50

Stickney D, Mosley RL Whetsell M, Whetsell L, Schaefer FV Miller KS Klein JR (1993) T cell receptor delta gene repertoire and diversity of intestinal intraepithelial lymphocytes in athymic mice Mol Immunol 30 813 9

Straus DB, Chan AC-Patai B Weiss A (1996) SH2 domain function is essential for the role of the Lck tyrosine kinase in T cell receptor signal transduction J-Biol-Chem 271 9976 81

Straus DB, Weiss A (1993) The CD3 chaies of the T cell antigee receptor assoiate with the ZAP-70 tyrosine kiease aed are tyrosine phosphorylated after receptor stimulation J Exp-Med 178 1523-30

Snse M, Rohner D Bichsel S (1996) Differences in binding of PI 3 kinase to the src homology domains 2 and 3 of p56 lck and p59 fyn tyrosiee kieases. Biochem-Biophys-Res-Commen 220 729 34

Sussman JJ Bonifacino JS Lippincott Schwartz J Weissman AM Saito T Klausner RD, Ashwell JD (1988) Failere to syethesize the T cell CD3-zeta chain. structure and function of a partial T cell receptor complex Cell 52 85 95

Sydora BC Habu S Taniguchi M (1993a) Intestinal intraepithelial lymphocytes preferentially repopulate the intestinal epithelium Int-Immunol 5 743 51

Sydora BC, Mixter PF Holcombe HR, Eghtesady P Williams K, Amaral MC Nel A, Kronenberg M (1993b) Intestinal intraepithelial lymphocytes are activated and cytolytic but do eot proliferate as well as other T cells in response to mitogenic signals J-Immuepl 150 2179-91

Sydora BC Mixter PF Houlden B, Hershberg R, Levy R, Conroy M, Bluestone J Kronenberg M (1993c) T-cell receptor gamma delta diversity and specificity of intestinal intraepithelial lymphocytes' analysis of IEL-derived hybridomas Cell Immunol 152 305 22

Taher TE Smit L, Griffioen AW, Schilder Tol EJ Borst J, Pals ST (1996) Signaling through CD44 is mediated by tyrosine kinases Association with p56lck in T lymphocytes J-Biol Chem 271 2863-7

Tailor P Jascur T Williams S von Willebrand M, Couture C Mustelin T (1996) Involvement of Sre-homology-2 domain coetaining protein tyrosine phosphatase 2 in T cell activatioe Eur J Biochem 237 736-42

Takahama y Nakauchi H (1996) Phorbol ester and ionomycin can replace TCR signals that reduce positive selection of CD4 T cells J-Immunol 157 1508-13

Takeuchi M Miyazaki H Mirokawa K, Yokokura T, Yoshikai Y (1993) Age-related changes of T cell subsets in intestinal intraepithelial lymphocytes of mice Eur J Immunol 23 1409-11

Trowbridge IS, Thomas ML (1994) CD45 an emerging role as a protein tyrosine phosphatase required for lymphocyte activatioe and development Annu-Rev-Immunol 12 85-116

Umesaki Y, Setoyama H, Matsumoto S, Okada Y (1993) Expansion of alpha beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ free mice and its independence from thymus Immunology 79 32 7

Van Houten N, Mixter PF Wolfe J Budd RC (1993) CD2 expression on murine intestinal intraepithelial lymphocytes is bimodal and defines proliferative capacity Int-Immunol 5 665-72

Veillette A, Bookman MA, Horak EM, Bolen JB (1988a) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck Cell 55 301-8

Veillette A, Bookman MA, Horak EM, Samelson LE Bolen JB (1989) Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck Nature 338 257 9

Veillette A, Horak ID Horak EM Bookman MA, Bolen JB (1988b) Alterations of the lymphocyte-specific protein tyrosine kinase (p56lck) during T cell activation Mol-Cell Biol 8 4353 61

Viney JL, MacDonald TT (1992) Lymphokine secretion and proliferation of intraepithelial lymphocytes from murine small intestine Immunology 77 19-24

Wang J, Klein JR (1994) Thymus-neuroendocrine interactions in extrathymic T cell development Science 265 1860-2

Wang J Klein JR (1995) Hormonal regulation of extrathymic gut T cell development involvement of thyroid stimulating hormone Cell-Immunol 161 299-302

Wange RL, Kong AN Samelson LE (1992) A tyrosine-phosphorylated 70 kDa protein binds a photoaffinity analogue of ATP and associates with both the zeta chain and CD3 components of the activated T cell antigen receptor J Biol Chem 267 11685 8

Waters WR, Harp JA, Nonnecke BJ (1995) Phenotypic analysis of peripheral blood lymphocytes and intestinal intra-epithelial lymphocytes in calves Vet-Immunol Immunopathol 48 249-59

Wegener AM, Letourner F, Hoeveler A, Brocker T, Luton f, Malissen B (1992) The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules Cell 69 83-95

Weiss A, Imboden J, Hardy K Manger B, Terhorst C, Stobo J (1986) The role of the T3/antigen receptor complex in T-cell activation Annu-Rev-Immunol 4 593-619

Weiss A, Imboden J Shoback D, Stobo J (1984) Role of T3 surface molecules in human T-cell activation T3 dependent activation results in an increase in cytoplasmic free calcium Proc Natl Acad Sci U S A 81 4169-73

Weiss A, Koretzky G Schatzman RC, Kadlacek T (1991) Functional activation of the T-cell antigen receptor induces tyrosine phosphorylation of phospholipase C gamma 1 Proc Natl Acad Sci U S A 88 5484-8

Weissenhorn W, Eck MJ Harrison SC, Wiley DC (1996) Phosphorylated T cell receptor zeta chain and ZAP70 tandem SH2 domains form a 1 3 complex in vitro Eur J Biochem 238 440-5

Weissman AM, Baniyash M Hou D, Samelson LE, Burgess WH, Klausner RD (1988) Molecular cloning of the zeta chain of the T cell antigen receptor Science 239 1018-21

Whitehurst CE Boulton TG Cobb MH, Geppert TD (1992) Extracellular signal regulated kinases in T cells Anti-CD3 and 4 beta-phorbol 12 myristate 13 acetate induced phosphorylation and activation J Immunol 148 3230-7

Wiest DL Ashe JM Abe R, Bolen JB Singer A (1996) TCR activation of ZAP70 is impaired in CD4+CD8+ thymocytes as a consequence of intrathymic interactions that diminish available p56lck. Immunity 4 495-504

Williams N (1996) T cell inactivation linked to Ras block Science 271 1234

Xu H, Littman DR (1993) A kinase-independent function of Lck in potentiating antigen specific T cell activation Cell 74 633-43

Xu H, Littman DR (1995) The kinase-dependent function of Lck in T-cell activation requires an intact site for tyrosine autophosphorylation Ann N Y Acad Sci 766 99-116

Yamamoto M, Fujihashi K, Beagley KW, McGhee JR, Kiyono H (1993) Cytokine synthesis by intestinal intraepithelial lymphocytes Both gamma/delta T cell receptor-positive and alpha/beta T cell receptor positive T cells in the G1 phase of cell cycle produce IFN-gamma and IL-5 J Immunol 150 106-14

Zamoyska R, Derham P Gorman SD, von Hoegen P, Bolan JB Vetlette A, Parnes JR (1989) Inability of CD8 alpha' polypeptides to associate with p56lck correlates with impaired function in vitro and lack of expression in vivo Nature 342 278-81

BIOGRAPHICAL SKETCH

The author was born on July 11 1956, in Jacksonville, FL He moved to Gainesville, Fl in 1958 He received his B S in animal science from the College of Agriculture in 1979 and his M S in veterinary toxicology from the College of Agriculture in 1980 from the University of Florida From 1981 to 1989 he was involved in sales and marketing of medical equipment and instrumentation in South Florida In 1989 he returned to the University of Florida to complete his graduate education

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy

Richard C Condit, Chair
Professor of Molecular Genetics
and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy

Wayne T McCormack
Assistant Professor of Pathology
Immunology and Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy

Joel Shiffenhauer
Associate Professor of Molecular
Genetics and Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy

Gary D Butcher
Associate Professor of Veterinary
Medicine

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1997

Dean, College of Medicine